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SERUM TREATMENT OF LOBAR PNEUMONIA. A STUDY IN IMMUNOLOGY.

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THE results of a laboratory inquiry into the nature of the immunity reaction in animals, to pneumococcal infection have been presented in two communications published by the Royal Society in 1925. Observations have been made since then on the natural development of anti-pneumococcal immunity in patients suffering from lobar pneumonia, and combined passive and active immunity has been studied in adult cases of the disease treated with (Felton) concentrated anti-pneumococcal sera.

THE NATURAL IMMUNITY REACTION IN UNTREATED CASES OF LOBAR PNEUMONIA.

The development of immunity in the course of lobar pneumonia affords a classical example of the reaction of the human or animal host to bacterial invasion. There is general agreement that crisis in lobar pneumonia is associated with the sudden appearance and rapid increase of substances in the blood, protective against the homologous pneumococcal infection ; the opinion is current that serum immunity is low in complicated and prolonged cases and may be absent altogether in those which prove fatal.

Amongst recent workers may be cited Baldwin and Cecil (1926) who correlated the disappearance of pneumococci from the blood in severe lobar pneumonia with the appearance of antibodies. Sia, Robertson and Woo (1928) measured the " pneumococcocidal " power of the blood in seventeen cases of lobar pneumonia. They concluded that this test gave results nearly identical with those afforded by mouse protection. Bactericidal substances were lacking in a patient who died on the ninth day of disease. Empyemata

developed in two other cases, one with high, the other with a rather low degree of serum immunity. The work of Bullowa (1929) and of Sabin (1930) should also be cited and Cole's early studies (1917) on the agglutinin titre in cases treated by immunizing sera.

EXPERIMENTAL.

A little group of nine cases of pneumococcal lobar pneumonia in adults was studied by the writer, using the mouse protection test to decide, if possible, the relation between serum immunity and the clinical course of pneumonia, with particular regard to relapse, complication, sequelæ or even a fatal ending.

Samples of the patient's blood-serum were collected daily. The mice

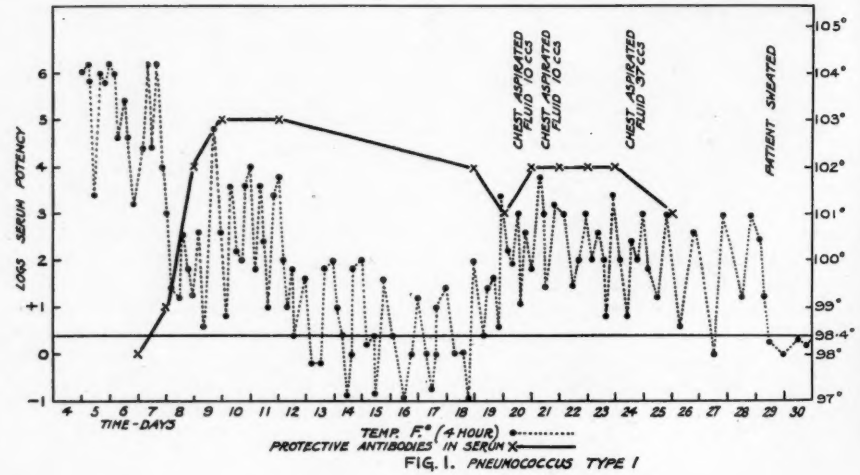

FIG. 1. PNEUMOCOCCUS TYPE I

received 0·2 c.c. of serum intraperitoneally over-night and 0·5 c.c. of decimal dilutions of a 16-hour blood broth culture next morning. Survival of the animals was held to be a measure of the protective power of the serum. The minimal lethal dose of every strain of pneumococcus studied was confirmed, in each experiment, by means of control mice, which received inocula of culture but no serum.

It is impossible to represent, in arithmetic progression, on a single figure, the observed wide variation in serum potency. The practice of decimal dilution of pneumococcal cultures is, however, directly adaptable to the expression of the numerical results on a logarithmic scale. Accordingly, in all diagrams, the figures given in the left margin are the characteristics of the logarithms of the number of minimal protective units in standard doses of 0·2 c.c. of serum: hence 0 represents one minimal protective dose; 6, one million doses. The protective power of serum samples, at successive intervals of time, is chartered

as abscissæ, the time in hours and days, as ordinates. The use of a logarithmic scale causes small differences in protective power at the bottom of the scale to be disproportionately enhanced in graphic representations, whilst large differences at the summit of the curve are minimized.

An inductive period of four or five days follows the rigor which usually marks the onset of pneumonia in a typical case. Protective power first develops on the fifth or sixth day and increases rapidly for a day or two. The curve shows an abrupt linear rise to a maximum on the eighth or ninth day, when the critical fall in temperature with striking amelioration of the patient's condition, known as "crisis," usually takes place.

The course of these events may be altered or delayed. Fig. 1 displays the clinical and serological picture in a severe case of lobar pneumonia in an adult man.

The temperature record indicates that the main infection was combated by the end of the sixth day, when protective antibodies were just beginning to appear in the blood. The balance of infection and resistance at this stage must have been nearly equal, for a minor relapse occurred, despite the rapid increase in protective potency of serum samples. The temperature rose again on the twentieth day to 101·5° F. and signs of fluid were detected. Aspiration was performed on successive days, 57 c.c. in all being withdrawn. On the twenty-ninth day the patient sweated and the temperature fell precipitately to a normal level.

An illustration is thus afforded of sudden development and rapid increase in serum immunity and of a persistently high concentration of protective substance; this surplus, however, by no means prevented the development of a fresh manifestation of pneumococcal activity, namely empyema thoracis. The general thesis is therefore confirmed, that close relationship exists between the clinical course of lobar pneumonia and serum immunity, but that the development of complications is independent.

Brief reference may be made to Fig. 2, picturing a struggle yet more hardly won by a patient against pneumococcus Type 3 infection. Here only the merest traces of protective power could be found on the sixth day and after, and the temperature remained high. A severe relapse followed, but the protective titre crept slowly up to the point of neutralization and there was a normal crisis. A complication akin to empyema, namely pericarditis, developed despite the presence of circulating antibodies.

Fig. 2 illustrates, in the human subject, the experimental observation (Armstrong, 1925) that protective response follows further antigenic stimulus, applied at any time during the inductive phase and the phase of rising immunity, provided that stimulus be not excessive. In severest cases, the protective mechanisms are overwhelmed and death ensues from the gravity of the infection itself, but this is not invariable. Thus in one of the group of 9 cases studied, protective antibodies appeared on the sixth day and immunity rose rapidly to a high level on the eighth day, when death occurred from heart failure. The patient, an enfeebled man, could not support the mechanical difficulties imposed by consolidation of both bases and a pre-existing adherent pericardium.

The substantial immunity represented by the high plateau of the curve, persisting long after crisis, is a characteristic feature of cases of pneumonia of

every grade of severity which recover, as well as of the reaction to adequate doses of pneumococcal vaccine in the experimental animal. An exception to

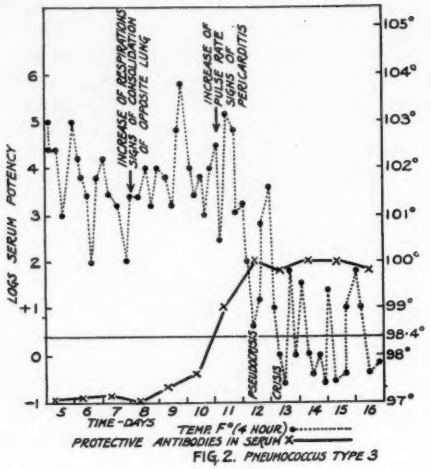

FIG. 2. PNEUMOCOCCUS TYPE 3

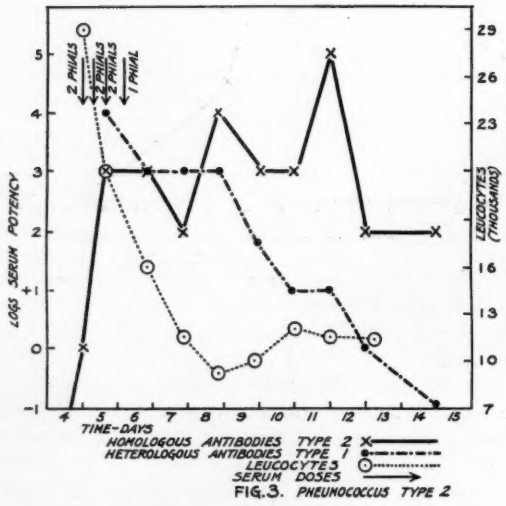

FIG. 3. PNEUMOCOCCUS TYPE 2

this general rule was found in cases in which generalized bronchitis accompanied the pneumonia; in these, temperature fell slowly by lysis, or fluctuated for some days and the amplitude of the immunity curve varied greatly.

THE IMMUNITY REACTION IN CASES TREATED WITH SERUM.

As described in a previous paper (Armstrong and Johnson, 1931) 26 early or severe cases of lobar pneumonia were treated by concentrated anti-pneumococcal sera. Four only of the treated cases could be selected for immunological study on account of the disturbance to the patients involved.

The salient features of the immunity curve are represented in Fig. 3, depicting events in a case of Type 2 pneumococcal infection treated with Felton concentrate; each phial of serum contained 10,000 units each of Type 1 and Type 2 antibodies. Treatment was begun at the end of the fourth day. Natural immunity was possibly on the point of appearance on this day, titra-

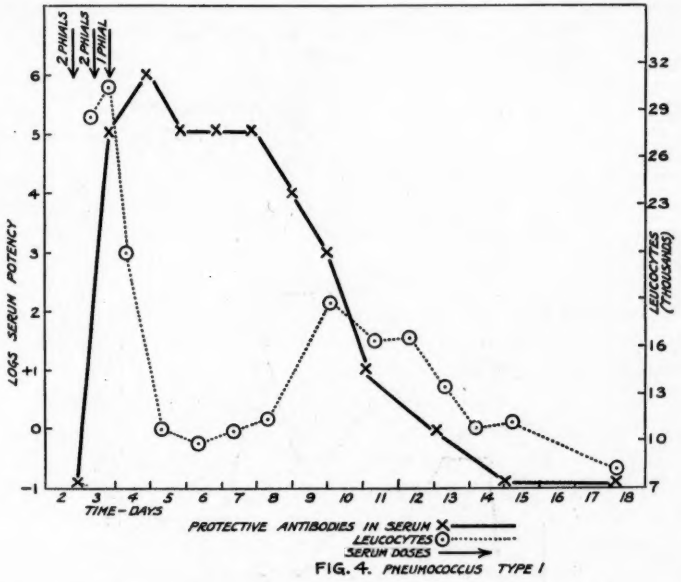

FIG. 4. PNEUMOCOCCUS TYPE I

tion of a sample of the patient's serum, collected before treatment, showed slight protective power.

Passive immunity falls in the 24 hours or more following the last intravenous inoculation of serum, at which time the loss is arrested by actively produced antibodies entering the circulation during the fifth to the seventh days. Titre rises steadily during the eighth day, falls on the ninth, and increases again on the eleventh day to a high maximum which is not maintained.

These features must be stressed, for they were nearly duplicated in two other cases studied. In one, due to Type 1 pneumococcus, treatment was begun on the third day of illness, when serum samples were devoid of protective power. The exhaustion of passively introduced antibodies was rapid, so that the immunity curve fell steeply to a low level; the surge of active immunity was

correspondingly emphasized, reaching a maximum on the eighth day as before. There was likewise a secondary peak on the eleventh day.

The immunity curve was similar in a third case, due to Type 2 pneumococcus, clinically severe and with extensive double pneumonia. The secondary peak on the eleventh day was not observed and immunity was low on the fourteenth day of the illness, when observations ended.

In this third case, the curve of disappearance of heterologous anti-pneumococcal substances, derived from the divalent Felton concentrate, was identical with that shown in Fig. 3. Characteristic features of this curve are the rapid reduction in serum titre in the first 24 hours, the period of three days during which changes are slight and a third phase of comparatively rapid loss of protective power to the point of disappearance ten days after intravenous injection. Glenny and Hopkins's (1922) work is specially apposite to these observations.

The appearances in Fig. 4 are at first sight anomalous. The highest point in the curve appears soon after passive immunization, there is then an uninterrupted fall in protective potency. This case was treated very early, 44 hours from the time of onset, and, symptomatically, was completely aborted, although physical signs of consolidation developed in due course. It is suggested that two doses of concentrate sufficed to stifle the nascent penumococcal infection and that the final dose provided an actual surplus of antibody, which appears as a peak at the beginning of the immunity curve. The active immunity response to the aborted antigenic stimulus was feeble and, in all probability, merged with and reinforced the curve of disappearing passive immunity. This conclusion follows from the fact that immunity levels, throughout the curve, were higher and protective power disappeared more slowly, than in the curve of wastage of heterologous antibodies traced in Fig. 3.

DIFFERENCES IN THE ACTIVE IMMUNITY CURVE IN TREATED AND UNTREATED CASES.

The curve of active immunity following passive immunization by administration of anti-pneumococcal concentrate, seems to differ from the natural response in untreated cases, in the sharp peak which marks the maximum level of active immunity. This effect is enhanced by the rather rapid loss of protective power (up to one hundredfold), which follows during the next day or two. These differences contrast with the comparatively slight loss of serum potency and the long, sustained plateau of the immunity curve in untreated cases. The active immunity in treated cases seems, therefore, inferior, so far as evidence limited to four experiments is of significance.

This result of passive immunization might cause misgiving, did it happen that relapse or recurrence was prone to occur in serum treated cases. There was no such experience. It appears, therefore, that even in the case treated very early (Fig. 4) in which demonstrable serum immunity soon vanished, the patient remained resistant against infection. The sputum in this case harboured virulent pneumococci, of the strain responsible for the pneumonia, a fortnight after clinical manifestations of the disease were over, yet convalescence was uninterrupted.

A SIMPLE ALTERNATIVE TO THE IMMUNITY TEST.

It may be objected that the course of immunity can be studied with success only in the laboratory, and that the titration of protective substances in a patient's serum is a laborious and slow procedure, which can scarcely afford a guide to treatment in the acute stage of a case of lobar pneumonia.

The leucocyte count is a simple alternative test, affording immediate information on the reaction of the patient to pneumococcal infection. A low count early in the illness is unquestionably unfavourable, indicating poor response or a highly virulent infection. Administration of serum has been promptly followed by leucocytosis in several severe cases. Fig. 3 indicates that the leucocyte count is a faithful index of the development of immunity, a favourable reaction being always accompanied by a fall to normal numbers. A transient moderate increase is frequently found seven days after inoculation of the concentrate and is possibly a serum effect; other symptoms of serum sickness are exceptional.

My gratitude is due to the Medical Research Council for material support in conducting this inquiry, and to my colleague, R. S. Johnson, for much valuable assistance.

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MUCUS SECRETION IN ACUTE EXPERIMENTAL INFLAMMATION OF THE COLON, AND OTHER MUCOUS MEMBRANES OF THE CAT; HISTOLOGICAL CHANGES.

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APPLICATIONS of appropriate dilutions of mustard oil in olive oil to the mucous membrane of the cat's colon result in a complete emptying of the goblet-cells (Florey, 1930). The latter, when the mucosa is in an "unstimulated" state, slowly expel their mucin content, as was shown with a Thiry fistula of the colon. If these cells are subjected to irritation by any agent capable of producing inflammation they discharge their mucin in the course of a few hours. It is with the changes involved in this rapid secretion that this paper deals.

EXPERIMENTAL.

The main tissue in which the reactions have been studied is the colon of the cat, though observations on some other mucosæ are included.

The cats were anæsthetized with chloralose administered intravenously. The abdomen was opened longitudinally and the colon pulled out. A loop of string was tied around the colon just proximal to the point at which the vessels are applied directly to the colonic wall. Another was placed about 6 cm. nearer the ileocolic sphincter. Any fæces contained in the colon between the strings was evacuated through a small transverse opening proximal to the loop before tying the second string. Mustard oil dissolved in olive oil was then injected into the colon by a hypodermic syringe through a wide-bore needle, till a moderate distension of the gut was produced. The intestine was then replaced, the abdomen sewn up, and the animal left for the duration of the experiment. In nearly all experiments the colon when opened contained large amounts of "egg-white" mucus (as has been previously described); the mucosa was uniformly red, and in the more severely inflamed cases petechial hæmorrhages were found.

Histological Methods.—Before beginning the manipulations for the preparation of the colonic loop a small piece of mucosa, free from muscle, was cut out, pinned on cork and fixed. At the end of the experiment the inflamed mucosa was treated similarly. The fixative employed as a routine was Heidenhain's "Susa" (Heidenhain, 1916). Sections prepared from the fixed tissue have been stained in Heidenhain's iron-alum hæmatoxylin, and duplicates additionally stained in Mayer's muci-carmine as a specific stain for mucin.

* With a grant from the Medical Research Council.

The normal control sections of the colon were cut as far as possible vertical to the mucosa.

RESULTS.

Colonic Mucosa.

A short description of normal mucosa is here given to facilitate comparisons with the inflamed tissue. While all of the crypts were rich in goblet-cells, the total numbers and the proportion of goblet-cells to resting epithelium varied from animal to animal in the series. In one cat, for example, where the crypts appeared to be exceptionally full of goblets a count of the cells throughout the whole crypt length gave an average figure for three crypts of 50 goblet-cells to a total of 125 epithelial nuclei. The epithelium of the surface between the openings of the crypts was always relatively free from goblet-cells. Occasionally one, rarely two, could be found, but in the majority none were present in a row of 10 to 15 surface cells. The bases of the crypts were frequently slightly dilated and their cavities full of mucin strands. The epithelium in such bases showed several goblet-cells with a small content of mucin, while the remainder of the cells were cuboidal in shape and mucin-free. But this was by no means a constantly occurring picture in the bases of the crypts. Frequently appearances very similar to those of the upper portions of the glands were present, *i. e.* the entire semicircle of the base was found studded with fully formed goblets, each of almost maximal size and with little diminution in their long diameters. The appearance of these bases rich in goblets is emphasized here because of the striking contrast it forms with the picture after mustard oil stimulation (Fig. 1*a*).

The typical goblet-cell of the crypt, whatever its position, was nearly round in shape, except in those cases where the ruptured theca had allowed the mucin to begin its exit from the cell. Goblets with oval apices and a definite stem were rarely seen. The mucin in iron-alum hæmatoxylin sections showed only as clear, unstained spaces, but with muci-carmine it appeared as fine threads of pink interlaced into a net-like mass with meshes of lighter staining. Where the goblet was discharging, the intracellular strands could be seen continued as a tangled skein into the lumen of the crypt. It was not possible to make a distinction in the staining between the mucin within an intact goblet and that of a ruptured goblet, or that discharged into the lumen. In the fully-formed goblet-cell some distortion of the nucleus was invariably present. The most pronounced change was a compression of the nucleus into a thin crescent or triangle, staining almost jet-black. These crescentic and triangular nuclei in many cases lay under the exact centres of the bases of the balls of mucin, flattened against the limiting membrane of the cell. When the rupture of the theca with discharge of mucin had relieved the pressure sufficiently the nucleus recoiled, for goblets with only a small plug of mucin protruding from their still swollen interiors showed vesicular nuclei of the epithelial cell type. This recoil of the nucleus, however, is not necessarily very rapid, for similar cells with compressed crescentic nuclei could be found, in spite of ruptured thecas and beginning extrusion of the mucin. The nucleus of the neck cell—the elongated non-goblet epithelium—did not differ from the nucleus of the

cell of the crypt base, the short, cuboidal cell with very little cytoplasm above the upper pole of the nucleus. In both cases the nuclear membrane was clear-cut and sharply stained, there were either one or two nucleoli, and the chromatin formed very delicate threads in a relatively large amount of unstained nuclear sap. A variant of this vesicular nucleus of the epithelial cell did occur occasionally. The staining was intensely dark, so much so that the nucleolus was barely distinguishable, but the size of the nucleus was not altered, and there was no evidence of compression. No difference in the cell bodies could be correlated with these dense nuclei. Mitotic figures, in all phases, could be found in the normal crypts. While rarely occurring in the extreme basal cells, they were much more frequent near the base than high up among the neck-cells. The mitotic cell was never a mucin-containing cell. The cell body was usually spherical and somewhat swollen, and its position was nearly always removed from the basement membrane and near the crypt lumen, as though the process of mitotic division required more room than was available along the closely set basal portion of the epithelial row. The number of mitotic figures seen in sections of the normal colon mucosa varied considerably in different cats. One cat, for example, showed an exceptional profusion of mitoses. The section through the mucosa was tangential instead of vertical, and in the two bottom rows of cross-sectioned crypts, one or two mitotic figures could be found in every second or third crypt. The mucin content of this mucosa seemed of average richness.

The histological appearances of the mucosa following the application of mustard oil varied in degree with the concentration of the oil and the length of time of application. In the 19 cats in which colon experiments were done, the strength of the mustard oil varied from 2 drops in 25 c.c. of olive oil to 40 drops in 25 c.c. The time during which the oil remained in the colon pouch varied from $3\frac{1}{4}$ hours to 20 hours.

The desquamation and necrosis produced by the application of the concentrated oil made weaker doses desirable, and in the majority of the experiments 4 or 8 drops in 25 c.c. were the concentrations chosen. $3\frac{1}{4}$ to 7 hours' application were the most common periods of time. In an experiment in which a dose of 8 drops in 25 c.c. was left in the colonic pouch for $3\frac{1}{2}$ hours, the pouch on opening showed a large amount of "egg-white" mucus and the mucosa was uniformly red. There were no petechiæ.

The most striking microscopical changes in this animal were the almost total absence of goblet-cells and the marked dilatation of the lower part of the crypts, together with an extreme flattening of the epithelium (Fig. 1, *b* and *c*).

A low-power view of the vertically cut crypts showed them as widely dilated fusiform spaces. Among these not a single goblet-cell could be demonstrated, using the muci-carmine stain. Two or three small balls of mucin were seen in the neck-cells, and strands of pink mucin were present in some of the dilated crypts, but these—with the exception of the exudate on the surface—were the only positively staining materials to be found. The epithelium of these dilated crypts was cuboidal. It appeared as a closely set even row of cells, with round or oval nuclei similar to the normal nuclei, and with light vesicular staining, but in which the cytoplasm was reduced to a depth of barely half the nuclear diameter. The iron-alum hæmatoxylin gave

this cytoplasm a faint grey tinge, and the only traces of mucin to be found were one or two thin strands at the margin of a few of these cells. The flattened row was quite uniform, and the outer cell margins formed a continuous and even line indicating that goblet- and epithelial cells were both reduced in height to the same extent.

In certain crypts the epithelium was even more altered. These were widely dilated, though in most cases the lumen no longer contained mucin, and the dilatation was accompanied by a flattening of the epithelium to the point of nuclear distortion, the crypts showing a lining of almost squamous epithelium. Gradations between these squamous cells and the cylindrical epithelium of the mouths of the glands were present, but even in the upper part of the necks of the crypts cuboidal or very short cylindrical cells were the rule. In certain experiments in which the exhaustion of the mucin content was not so pronounced, the lining of cuboidal epithelium merged gradually with cells of more normal height as the mid-portion of the crypt was reached. In such a crypt the row of cells showed every transition, from the flat squame to the tall cylindrical cell, the latter with or without an intracellular mucin ball.

The scanty intracellular mucin to be found by a search of the section occurred in three forms. In the first place, there were occasional neck-cells in which small amounts of mucin were present. These resembled the normal type in everything but size, and were probably partially discharged goblets. The second type were cuboidal cells with an extremely small strand or plug of mucin occupying a part of the restricted cytoplasm. These cells were demonstrably incompletely exhausted goblet-cells, for the intracellular plug was continuous with strands of mucin on the surfaces of the cell and lying free within the crypt. In the third type a small cluster of tiny granules, staining faintly pink, lay next to the nucleus, with apparently intact cytoplasm above the cluster. The appearance and position of the granules suggested the beginnings of mucin formation within the cell. If, on the contrary, they did represent incompletely emptied goblets, then a trace of mucin at the bottom of the goblet-cup must have been entrapped during the process of discharge.

The effect of the mustard oil upon the goblet-cells in the necks of the crypts was similar, except for the fact that the discharged cell remained cylindrical.

The surface epithelium between the crypt mouths, in this cat, had suffered a certain amount of desquamation. The sections showed a layer of mucus, in which a number of lymphocytes and red blood-cells could be found, closely adherent to the mucosa, and beneath this layer some of the surface cells had disappeared. This desquamation of the surface epithelium varied from animal to animal. When the concentration of mustard oil was high there was extensive necrosis of the upper part of the mucous membrane, and a mass of cellular detritus mixed with mucus formed the superficial layers of the section (Fig. 1, *d*). Two cats with relatively huge doses of 40 and 30 drops of mustard oil respectively in 25 c.c. olive oil, applied for 4 and 5 hours, showed this necrosis of the upper half of the mucous membrane. But where the cells were killed by the high concentration of the irritant the emptying of the goblet-cells was incomplete. In the ragged remains of the top half of such a crypt, where individual cells were hardly recognizable, round balls of mucin were numerous,

each enclosed by a still intact theca. This non-emptying of the cells could be observed also in the case of milder doses, provided any surface desquamation was present. In the cat first described, for example, in which the dose of mustard oil was 8 drops in 25 c.c., the small intestine showed desquamated tips of villi lying free in the mucous exudate, and in many of these perfectly preserved goblet-cells were to be found.

Mitosis.

There was a wide variation in the mitotic figure content of the normal controls. One animal has been mentioned as showing an exceptional number of cells in mitosis. In other normals, however, mitoses were very scanty, and could only be found after prolonged search.

In most experiments a definite increase in the number of mitotic figures occurred after mustard-oil stimulation, and in certain experiments the increase over the normal was very marked (Fig. 6). As in the normal controls, the cells in mitosis were distributed through the entire length of the crypt, except that they were rare in the very basal cells and were not found beyond the crypt mouths. Mitosis was never observed in the surface epithelium. Again, as in the normal, the epithelial cell in mitotic division lay lumenwards from the basement membrane, was rounded up and exhibited all stages of mitosis, though late telophase was somewhat rare. A common finding was a cluster of mitotic cells closely grouped together in the lower third of the crypt. One cat showed a number of such clusters in successive crypts. This portion of the crypt was definitely the most usual site for mitoses. The mitotic cells never showed any intracellular mucin when stained with muci-carmine.

In several of the experiments irregular mitotic figures were observed, following mustard-oil stimulation. In one animal, in which mustard oil (4 drops in 25 c.c.) had been applied for 6 hours, the number of cells in mitosis was very high, and this irregularity was present in all (Fig. 4). The cells were large and pale, and showed chromatin clumps in the form of spheres, ovoids or short rods scattered haphazardly throughout the cytoplasm. The units of chromatin were occasionally clumped in one corner of the cell, but the more usual appearance was a scattered distribution. No signs of normal spindle formation were found. The cells in many cases bore a striking resemblance to those pictured by Ludford (1930) in experimental sarcomata and carcinomata of rats and mice. He found these abnormal mitoses in three transplantable sarcomata and in one transplantable carcinoma, and was able to show successive stages of the process in stained sections. He concluded from these that the first steps in the formation of chromosomes from the nucleus and their liberation through breaking down of the nuclear membrane occurred in the usual way, but that the achromatic spindle failed to develop, and its absence accounted for the wandering of the chromosome segments throughout the cytoplasm. Our examples did not show one feature which he pictures, viz. the concentration of the chromosomes at the periphery of a spherical cell. If Ludford's suggestion be accepted, the interruption of the cycle of events occurs at the point where the colloids of the cytoplasm undergo a change in state leading to the formation of the achromatic spindle.

Our material has not supplied any information as to their further development.

Effect of Pilocarpine.

In one animal (Cat 156) a series of 20 doses of pilocarpine (each 0·5 c.c. of 1/1000 solution) were given intravenously over a period of 4½ hours, at the end of which the animal died. There was not much mucus in the colon when opened, as it had been passed *per anum* during the course of the experiment, but the mucosa was very red and showed minute petechial hæmorrhages.

Microscopically the mucosa exhibited marked necrosis of the surface epithelium and an intense cellular reaction of polymorphonuclears throughout the lamina propria. The bases of the crypts showed considerable exhaustion of goblet-cells, but above the bases practically no epithelium remained; the crypts disappeared into the cellular mass of the lamina propria. The vessels of the submucosa were greatly engorged, and there was a moderate accumulation of polymorphonuclears in the submucosa and in the muscular wall. Repeated intravenous injections of pilocarpine, therefore, in the cat produce a marked inflammatory reaction throughout all the coats of the colon.

Restoration of Exhausted Goblet-Cells.

A series of experiments was done to determine whether the goblet-cells, after rapid exhaustion by mustard oil, could be readily restored.

Under ether anæsthesia and with aseptic precautions a loop of colon was prepared by tying off a portion with string ligatures, and filled with the solution of mustard oil in olive oil. Before injecting the oil a piece of the wall was excised to serve as a normal control, and any fæces present evacuated through the opening, which was then repaired. The abdomen was closed and the animal allowed to recover. At the expiration of 4 to 8 hours the colon was evacuated, under anæsthesia, through a second opening. Another strip of gut wall was removed for microscopical examination. This opening was sewn up as before and the string ligatures removed. None of the colons so treated showed any appreciable injury at the lines of ligature. After closing the abdomen a second time the cats were again allowed to recover from the anæsthetic and were kept in a warm room until they were killed—usually 24 hours after the second operation.

In 5 cats so treated, all but one exhibited marked exhaustion of the goblet-cells in the specimens removed when the mustard oil was evacuated (Fig. 5, *a* and *b*). The appearances were identical with those described in the preceding paragraphs and do not require further elaboration. The doses of mustard oil were varied in an attempt to procure maximal emptying of the cells, and in one animal there was considerable necrosis and erosion of the surface layer of the mucosa. In two other cats there was a similar superficial desquamation, but slight in degree. None of the animals in these recovery experiments showed the degree of exhaustion obtained in cats under continuous chloralose anæsthesia. In the recovery series there was a short period of anæsthesia only, and the bulk of mucus secretion took place in non-anæsthetized animals.

In three of the experiments in which there was a considerable degree of exhaustion of the goblet-cells after applying mustard oil for 6½ hours, 8 hours and 4¾ hours respectively, recovery with restoration of the mucin content was almost complete at the expiration of a further 24 to 48 hours (Fig. 5, *c* and *d*). A picture approximating the normal control, if not quite its equal in the total number of goblet-cells, was observed in each case. A study of the crypt bases in the three stages—normal control, exhaustion, recovery—furnished a striking contrast. In the normal control the columnar epithelium of the bases and necks gave the impression that almost every cell present was a goblet. In the exhausted stage the dilatation of the bases was very prominent. They showed as wide spaces lined with low cuboidal epithelium containing hardly a trace of intracellular mucin. Many mitoses could be found. In the recovery stage, within 24 to 48 hours, the whole length of the crypts exhibited numerous goblets once more—perhaps two-thirds the number of the normal control—and the bases, while still slightly dilated, showed a return to columnar epithelium. These cells presented a series of changes which indicate the method of re-formation of the secretion (Fig. 5, *c*). In the first stage a cluster of tiny granules, faintly pink, were collected together just above the nucleus. In the next stage the mucin, staining more deeply, was extended in thin strands from the upper pole of the nucleus to the free border of the cell. Then rather dense compact plugs could be seen completely filling, but not distending, the narrow cylindrical cell. The next phase showed the gradations of goblet formation, from the cell whose walls were only slightly bellied out by the secretion, through every sort of intermediate stage, to the fully-formed and rounded goblet. The largest and most distended of the goblets were always found at the crypt mouths or among the cells of the free surface ; below this point good specimens could be seen, but they were never the most striking. Many of the crypts in these recovering mucosæ contained thin strands of mucus. This was derived beyond doubt from the immature goblets during their phase of refilling, for in all of the stages just described cells could be found with streamers of mucin joining the secretion in the depths of the cell to the mass of secretion in the lumen. The mitotic figures had dropped to a number comparable with that of the normal control.

One cat in this recovery series presented a somewhat different appearance, due to the marked necrosis of the epithelium at the end of 5 hours' application of mustard oil. The goblet-cells were scanty where the epithelium was necrotic, but could be found in considerable numbers where intact cells were present. The animal was kept for three days after evacuation of the irritant, in an attempt to demonstrate complete restoration of the mucin content, but when microscopical sections were prepared, the submucosa was found to be greatly thickened by œdema, and in the whole of the mucosa it was hardly possible to find a single intact epithelial cell. The necrosis initiated by the mustard oil had obviously progressed to a point at which no epithelial cells were surviving.

The colonic epithelium was observed in a healing wound of 24 hours' duration. A section was prepared through the line of suture of the colon wall in this animal 24 hours after the suture was done. The section did not show healing in the mucosa itself, as there was not absolute apposition of the mucosal

fragments, but the colonic muscle had become firmly sealed by a line of fibrin. Where the mucosa had been divided and was somewhat ragged and torn, portions of crypts could be seen with goblet-cells in numbers still present in them.

The Stomach.

The effect of mustard oil upon the gastric mucosa was also investigated. The oil was introduced through a hole in the stomach-wall (which served also to provide a strip for the normal control), after ligating the œsophagus in the neck, and the duodenum just distal to the pyloric sphincter. Sections of the fundus and pylorus were prepared for study after varying periods of application of the oil—from $4\frac{1}{2}$ hours to $8\frac{1}{2}$ hours. The concentrations of mustard oil in olive oil varied from $3\frac{1}{2}$ drops in 25 c.c. to 32 drops in 25 c.c. For the most part 4 to 8 drops were employed. In every experiment of the series of six, the diminution in the stainable mucin of the cells was very small, even though in one animal the dose was so large as to cause necrosis and desquamation of the surface cells.

The stainable mucin of the normal cat's gastric mucosa is confined in the fundus to the surface epithelium and to the mucous neck-cells. In a vertically cut gland crypt, stained with muci-carmine, the surface epithelium and the neck cells show moderately pink mucin (these cells do not take the muci-carmine so well as intestinal goblets). They extend downwards in the gland, gradually becoming less elongated, until they are replaced by the chief and parietal cells which contain no mucin. In the closely set row of neck-cells every cell showed mucin. This was present in pink-staining strands rolled up in the form of a ball. Two or three of these lay side by side in the cylinder of cytoplasm between nucleus and cell border. In no case was any cell found identical with the typical goblet-cell of the colon.

The epithelial cells of the free surface were tall and cylindrical, with long oval nuclei, and their mucin content was always less than that of the neck-cells. A small ball of mucin was to be found in each cell near its outermost surface, but the intervening cytoplasm between mucin plug and nucleus showed a granular grey tint, frequently without a trace of pink. In the pylorus an exactly similar picture was present. The epithelium of the free surface showed a certain amount of mucin in the tips of the cells ; this increased in quantity in the neck-cells of the foveolæ until the latter gradually became shorter and merged with the clear cells of the pyloric glands, the latter being everywhere unstained by muci-carmine.

After the application of mustard oil the mucus-cells under low power seemed to exhibit an unchanged content of mucin. The rows of cells were even and uninterrupted and stained almost uniformly pink. But a closer examination with higher powers revealed the beginnings of mucin emptying. Half-way down the foveolæ a fusiform dilatation was occasionally to be found, and into this dilated part of the lumen a few neck-cells were on the point of discharge, their mucin plugs lying half in and half out of the cells. The fusiform dilatation was frequently full of the extruded secretion. But these changes required searching for, and were not present in all of the animals of the series.

The cells of the stomach are clearly much less liable to exhaustion of their mucin content when stimulated than the goblets of the colon. It should be emphasized that although there was but slight evidence of microscopical exhaustion of the mucus-producing cells, there had been an abundant macroscopical secretion.

There was no increase in mitosis after mustard oil. This was clear from a careful examination of the surface cells, the mucous neck-cells and the deeper chief and parietal cells of the gland tubules. It was possible to make a systematic search of all the epithelial cells in a strip of mucosa without discovering a single mitotic figure. In this the sections resembled exactly the normal controls.

Small Intestine.

Seven experiments were performed upon the small intestine. The concentration of mustard oil in olive oil varied from 1 drop in 150 c.c. to 8 drops in 25 c.c. The latter dose produced necrosis of the mucous membrane, and it was found, in general, that the response to mustard-oil stimulation was much more marked than in the colon. Smaller doses were therefore the rule in the majority of the experiments.

Sections of the normal small intestine showed on the whole very constant appearances (Fig. 2, *a*). The epithelial lining of the villi, whether the latter was composed of very long cells or those of medium height, was sprinkled with goblets, but the number of non-goblets always greatly exceeded the goblets. In one animal, for example, the goblet-cells were as few as 14 per 300 epithelial cells forming the circumference of a villus. In another a more average figure of 35 goblets to 300 cells was found. These isolated goblet-cells of the villi gave exceptionally clear-cut microscopical pictures. Whereas the cylindrical epithelial cell of the villus had an elongated thin nucleus, vesicular and lightly staining, the nucleus of the fully formed goblet was in its typical form crushed into a short crescent at the bottom of the goblet cup. The size of the goblet-cells showed a certain amount of variation, but in every case the ball of mucin was sharply outlined, usually round or oval, and where the cell was discharging could be seen flowing to the exterior through a sharp break in the cuticular border. This remnant of the cuticular border was clearly seen. It did not suggest in any way an artefact, such as the overlying of an epithelial cell by a superimposed goblet, as maintained by Bizzozero (Schaffer, 1927).

The goblets became concentrated at either side of the bases of the villi and were continued past the mouths into the crypts of Lieberkühn. Their numbers varied slightly in individual crypts, but they were plentifully sprinkled along the whole length of the crypts from mouth to base. As the epithelium became more cuboidal in the body of the crypts the goblet-cells became correspondingly smaller in their long axes, but they remained clear-cut and their numbers were not diminished. In the mid-portion of a crypt, for example, 8 goblets were found to 24 cells. The extreme basal epithelium rarely showed more than one or two goblets, but these were present occasionally, and were tall and fully formed.

Mitotic figures occurred with considerable frequency, being found at any point from just above the extreme base to the mouth of the crypt.

The effect of mustard oil was observed most clearly in the middle and basal portions of the crypts. The villi, it is true, in some of the animals showed a definite though small reduction in goblet-cells, but in the majority there was no reduction (Fig. 2, *b*). The goblets were always less in number at the bases of the villi, a situation in which they were concentrated in the normal control. In the mouths of the crypts and along the whole length of epithelium, from mouth to base, there was a considerable diminution in goblet-cells. Where the normal control showed the presence of 8 goblet to 24 epithelial cells, after mustard oil the proportion was reduced to 2 to 24, and in many crypts none at all were to be found in the whole of one side of the crypt. The bases usually were quite devoid of goblet-cells, but the dilatation of this portion, and the flattening of the epithelium, which were features of the colon, were not present here. The epithelium from mouth to base was of a short columnar type, and even where no goblets at all were to be found, the cells were hardly more cuboidal or flattened than in the normal. Goblet-cells in all stages of partial emptying could be found, down to cells with a tiny cluster of small granules just above the upper pole of the nucleus.

The intestinal villi were more sensitive to the mustard oil than the surface epithelium of the colon. In nearly every experiment there were signs of beginning desquamation of the epithelial lining of the villi, and in one cat, which had received a dose of 4 drops of mustard oil in 25 c.c. olive oil for a period of 7 hours, there was actual rupture of the epithelium, with a streaming out of the stroma-cells through the gap. The appearance was due to œdema of the lamina propria of the villi. In this animal, and in a second cat as well, numerous villi could be seen with a broad clear area separating the detached epithelium from the lamina propria (Fig. 2, *b*). The detached strip showed apparently healthy cells forming an even row where their bases had been separated from the basement membrane, and there were several goblet-cells scattered throughout the row. This œdema and detachment of the epithelium is probably the first stage of a process which ends in complete desquamation and necrosis of the villi.

The irritant effect of the mustard oil was manifested in another form in one animal, in which there was infestation with a coccidial parasite. This cat showed in the normal control the presence of numerous intracellular parasites (family Eimeriidæ*) in various stages of development in the tips of the villi. On examination, after 6¼ hours' mustard-oil application, the villi were almost free from the parasites; only one or two degenerate intracellular forms could be found.

In two experiments very low concentrations of mustard oil, 1 drop in 100 c.c. and 1 drop in 150 c.c. of olive oil, were employed to obviate this tendency to early œdema and desquamation. In this the experiments succeeded; the villi showed none of the degenerative changes described, while the reduction in goblet-cells as compared with the normal was marked. In the cat, with a dose of 1 drop in 150 c.c. applied for 7 hours, the entire circumference of one crypt base and half the body of the crypt showed only two partially exhausted goblet-cells—a reduction of 8 to 10 times from the normal control.

* We are indebted to Prof. Keilin for examining our slides and giving us his opinion of the parasite.

Mitoses were readily found in all sections of the intestine after mustard oil stimulation. As in the normal controls, they occurred anywhere in the crypt from the base to just below the mouth, but were concentrated especially about the lower third of the crypt. In one experiment the number of mitotic figures was definitely in excess over the normal. In the remaining experiments, however, it was extremely difficult to be sure of any increase. There was never any reduction.

Urinary Bladder.

Three experiments were done in which the urinary bladder was emptied through an incision and then filled with a solution of mustard oil in olive oil.

The doses were 4 drops, 3½ drops and 2 drops respectively in 25 c.c. of olive oil, and the periods of application were 3½ hours, 6 hours and 7 hours, after which the animals were killed for study. Only one normal control was taken, but it was clear from a microscopical examination that the transitional epithelium of the bladder mucosa possessed no mucin stainable by muci-carmine. The epithelial layer was 3 to 4 cells in thickness, and the cytoplasm of all the cells appeared a diffuse grey, and was slightly granular when stained with iron-alum hæmatoxylin and muci-carmine.

An occasional mitotic figure was seen in the basal layer of cells.

After the application of mustard oil the lamina propria and muscular wall showed extensive œdema and the vessels were greatly dilated. The surface of the mucosa was covered with a thin exudate, not containing many cells, and showing no colour with muci-carmine. In one cat the epithelial cells of the outermost layer were greatly swollen and granular, with dense, pycnotic nuclei, and were desquamating into the fibrinous shreds of the exudate. There was marked engorgement of all the vessels just below the basement-membrane.

In a second cat the desquamation of the surface epithelium had progressed a stage further. Only the two basal rows of cells remained; the others were missing, and as there was no exudate present, these had doubtless been separated before fixation. The same intense vascular reaction was present just below the basement membrane.

In none of the sections were mitoses prominent, and it was clear that no increase in mitotic cells had occurred in any of the experiments.

Gall-bladder.

One experiment was performed upon the gall-bladder. After excising a small portion of the wall for normal histology, the common duct was tied and the gall-bladder filled with mustard oil in olive oil (3½ drops in 25 c.c.). After 3½ hours the cat was killed.

In the normal control the columnar epithelium showed variation in height, the more cuboidal cells being at the tips of the mucosal folds, but in none of the cells was there any specific staining by muci-carmine. Mitoses were not found.

After mustard oil no changes in the staining with muci-carmine were seen. Inflammatory signs were present, but not marked. A thin exudate lay on

the surface, but the epithelium beneath showed only a trace of early desquamation in one part. The blood-vessels were moderately engorged, and there were red blood cells in the surface exudate. The epithelium throughout showed swollen cells. The cytoplasm was taller and more columnar than in the control and gave the impression of cellular œdema. No mitotic figures were found.

Uterus.

In one experiment the effect of mustard oil upon the uterine mucosa was investigated.

After excising one horn for normal histology the other horn was ligated in two places and injected with mustard oil in olive oil (4 drops in 25 c.c.) between the ligatures. The animal died at the expiration of $5\frac{1}{4}$ hours. The staining with muci-carmine in the normal control was slight. A few surface cells and a few cells lining the short gland crypts showed a very faint pink homogeneously distributed through the cytoplasm. There was a little secretion on the surface, and this secretion had taken on a slight pink colour. Mitoses were very scarce.

The effect of the mustard oil was limited to an early necrosis of the epithelium. Practically all the cells of the transversely or vertically cut tubules showed haziness of cell outline and a granular degeneration of the nuclei. The surface epithelium was partially desquamated. A few of the tubules showed slight dilatation and the presence of a small plug of secretion within the lumen. This plug took a very faint tint with the muci-carmine. In these dilated tubules there was a moderate flattening of the epithelium. No mitotic figures were found.

Vagina.

In one experiment the vagina was injected with mustard oil in olive oil (4 drops in 25 c.c.) and sewn up.

The animal died after $5\frac{1}{4}$ hours. The mustard oil had produced a little desquamation of the superficial layers of the stratified epithelium, the latter being only 2 to 3 cells in depth. The submucosa was œdematous. There was no colour with muci-carmine staining. No mitoses were seen.

DISCUSSION AND SUMMARY.

The histological appearances presented by the inflamed colon give some indication of the mechanism of discharge of the stimulated goblet-cell.

It is usually considered that in a slowly emptying goblet the walls of the theca collapse, and the cell appears thin and compressed among its more robust neighbours. Though this may occur during slow secretion, it does not appear to be the case in rapid exhaustion.

The process in the latter case can be reconstructed from the various stages observed in sections (Fig. 3, *a*, *b*, *c*, *d*, *e*, *f*, *g*).

Firstly through the stoma of the goblet there is an egress of concentrated mucus. With this freeing of the secretion the nucleus becomes round and

the goblet part of the cell less and less bellied out (Fig. 3, *b*). As the mucus is secreted more of the cytoplasm of the cell becomes revealed (Fig. 3, *c* and *d*), until the theca and its contents are represented by a small amount of concentrated mucus at the surface or on the tip of the cell (Fig. 3, *d*). Eventually even this disappears, and with it all trace of a thecal space. The goblet-cell now has the appearance of a columnar epithelial cell (Fig. 3, *e*). At this stage in some cells small granules of mucin can be seen in the cytoplasm confined to a region just above the nucleus. The surface of the cell appears to be completely re-formed—there is no trace of a stoma. With a continuance of the irritation the cell appears to lose cytoplasm and this applies equally to the epithelial cells. Firstly it becomes cuboidal (Fig. 3, *f*), and finally very like squamous epithelium (Fig. 3, *g*). There is no sign of desquamation of these cells or suggestion of death in the nuclear appearances.

An interesting point with regard to this exhaustion is the fact that the cells at the bases of the crypts undergo discharge and exhibit their cycle of changes before those nearer the surface. While the lower half of the crypts may be completely devoid of mucin, fully formed goblet-cells which have apparently discharged little mucin are present on the surface and at the mouths of the crypts. This is a phenomenon invariably encountered both in the small and large intestines. No adequate explanation can be offered.

In one of the animals a section of a healing incision of the colonic mucosa and muscular wall was available. This was of interest in that the goblet-cells in the immediate vicinity of the wound showed little if any diminution in their mucin content. It supports the view that goblet-cells react only to direct stimuli, and are not affected by those products of tissue injury which call forth an acute inflammatory reaction elsewhere.

At the same time the bases of the crypts become dilated (Fig. 1, *c*). This appearance might be due to a diminution in height of the living cells or to an accumulation of mucus. The former, however, is not sufficient in extent to account for all of the dilatation, and in these crypts it is rare to find more than a trace of mucus, most of it having passed to the surface. Here, again, no adequate explanation can be offered. A similar formation of "cysts" has been described by Sachs (1887) in the stomachs of dogs poisoned by tartar emetic, but he attributes them to retention of secretion. Sachs also noted the flattening of the epithelium (peptic cells) in the inflamed stomach.

The mucus-secreting cell of the stomach has different properties in its reaction to irritation. It has not proved possible to exhaust it in any way comparable to that which is so effective in the goblet. It appears, therefore, to be a much more resistant mechanism—perhaps a fortunate provision, since the stomach receives far greater chemical and mechanical insults than the colon.

No mucin was ever found in a dividing cell, in contradistinction to the assertion of Bizzozero (1889), who states that he observed this in normal mucosæ.

Majewski (1894) studied the colon in various animals for the results of pilocarpine administration. He mentions that Heidenhain and Klose were the first to show exhaustion of goblet-cells by this means. Though he himself could not produce complete exhaustion with the method, he noted that the

goblet-cells as they emptied assumed the appearances of normal epithelial cells. In many cases, especially at the bases of the crypts, cubical or even more flattened cells were seen.

He did not observe any increase in mitotic figures, but he noted a cons derable rise in the number of goblet-cells in animals which had been given large doses of pilocarpine some three or four days previously. He concludes that the goblet-cells can carry out their function repeatedly, and that ordinary epithelial cells may subserve this function of mucin production. The experiment detailed in this paper, in which pilocarpine was given in large amounts, is evidence that the drug acts in a similar manner to mustard oil. At any rate, all the signs of acute inflammation, including the sloughing of the surface of the mucosa, were present. Majewski, in his experiments, was really dealing with an inflammatory reaction.

A point of great interest is the ease and rapidity with which the colonic mucosa is able to repair itself, as the number of active mitoses shows (Fig. 6). These are in many cases greatly increased over the normal even in three hours. The same condition is found in the small intestine. A limited number of experiments on other mucosæ has failed to reveal a similar increase in so short a time. Schmidt (1896)* reports the presence of large numbers of mitoses in the gastric epithelium of dogs treated with alcohol. He does not give the length of time of his treatment. In the present experiments no mitoses have been observed in the stomach after the relatively short stimulation by mustard oil.

A note has been made of the irregular mitotic figures seen in several of the irritated colons (Fig. 4).

The re-formation of the goblets in the colonic mucosa takes place with considerable rapidity. Within two days a much exhausted mucosa is almost restored to normal. Owing to the large number of mitoses present at the end of the period of stimulation, it is impossible to say definitely whether the goblets are new formations, or are old mucin-producing cells elaborating new secretion. The appearances are consistent with the latter view, since every stage can be seen from the cuboidal cell containing a few granules of mucin to the fully formed goblet. Doubtless many new cells are present in addition. It is clear that the new cells originate primarily in the lower third of the crypt, for it is there that the great majority of mitotic figures occur. This strongly supports the view that regeneration of the surface epithelium is effected by an upward progression from below. As the elaboration of new secretion proceeds in the exhausted crypts, the cylindrical cells, whether they are newly formed or are the old exhausted goblets, show a definite secretion of mucus into the crypt during their progress towards the surface (Fig. 5, *c*). This secretion during the whole period in which the cell is becoming swollen into a goblet is a striking feature. It shows that the cell during this phase of immaturity is manufacturing its secretion at a rate greater than its discharge. The same process doubtless occurs under normal conditions, for there, too, the full-blown goblet of maximum size is only found towards the surface, and this same secretion of mucus is observed in goblets of intermediate size low down in the body of the crypt.

* It is not clear whether Schmidt is reporting his own experiments or is quoting from Ebstein and Sachs. If the latter is the case the irritant used was tartar emetic.

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DESCRIPTION OF FIGURES.

FIG. 1.—Mag. × 130. Stain: Iron-alum hæmatoxylin. (*a*) Normal crypts of colon. (*b*) Almost complete exhaustion of goblet-cells after mustard oil. Colon. Note numerous mitoses. (*c*) Dilated crypts after mustard oil, showing the extreme flattening of the epithelium. Colon. (*d*) Necrosis of upper part of colonic mucosa after mustard oil.

FIG. 2.—Mag. × 130. Stain: Iron-alum hæmatoxylin. (*a*) Normal small intestine. (*b*) Small intestine showing reduction in goblet-cells and the separation of epithelium of villi by œdema of connective tissue. After mustard oil.

FIG. 3.—Mag. × 930. Stain: Iron-alum hæmatoxylin and muci-carmine. A series showing stages of exhaustion of goblet-cells of the colon treated with mustard oil. (*a*) Normal fully-formed goblet-cell with triangular, compressed nucleus. (*b*, *c*, *d*) Successive stages of emptying. Note vesicular nuclei. All of the dark-staining material in the cytoplasm is mucin specifically stained by muci-carmine. (*e*) Cylindrical epithelium after complete evacuation of mucin. (*f*, *g*) Cuboidal and flattened epithelium from dilated crypts.

FIG. 4.—Mag. × 930. Stain: Iron-alum hæmatoxylin and eosin. Irregular mitotic figures in colon of animal treated for 6 hours with mustard oil.

FIG. 5.—Mag. × 310. Stain: Iron-alum hæmatoxylin and muci-carmine. Series showing exhaustion and partial recovery of goblet-cells in the same cat. Colon. (*a*) Base of normal crypt showing many goblet-cells. These obscure the non-goblet epithelium. (*b*) Cuboidal cells at base of crypt after mustard oil. Almost complete disappearance of mucin. (*c*) 24 hours after (*b*). Cylindrical cells at base of crypt showing stages of mucin re-formation. Note the discharge of mucin by immature goblet-cells. (*d*) 24 hours after (*b*). Upper portion of crypt showing completely regenerated epithelium and well-formed goblet-cells of smaller size than before irritation.

FIG. 6.—Mag. × 310. Stain: Iron-alum hæmatoxylin. Colon after mustard oil. The arrows point to the numerous mitoses.

(Microphotographs by H. P. Hudson.)

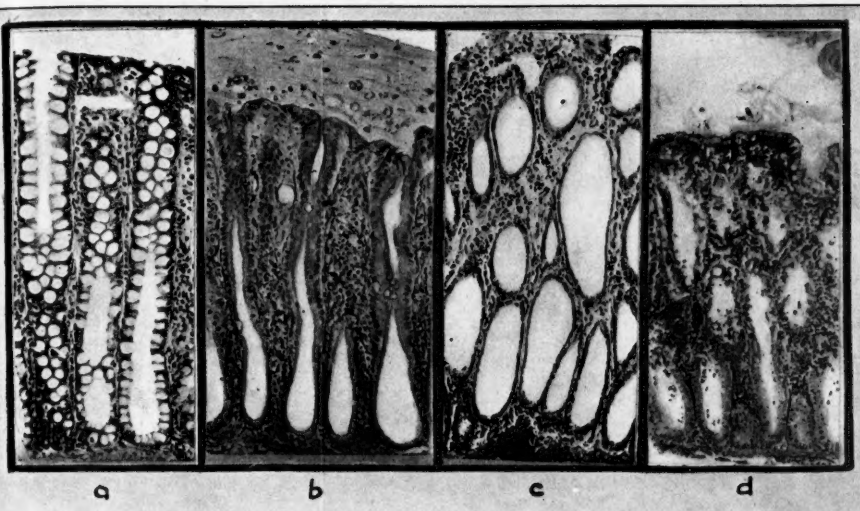


Fig. I

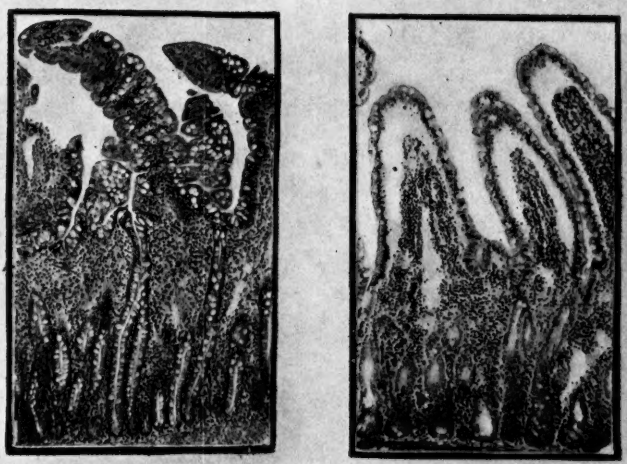


Fig. II

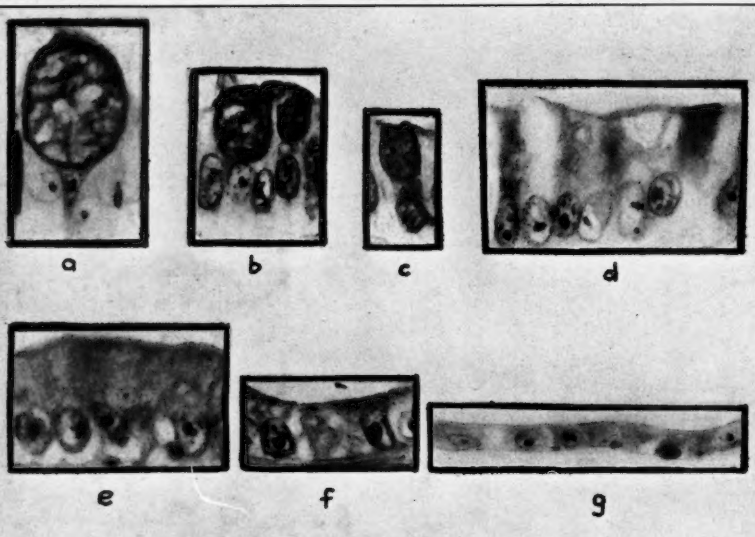
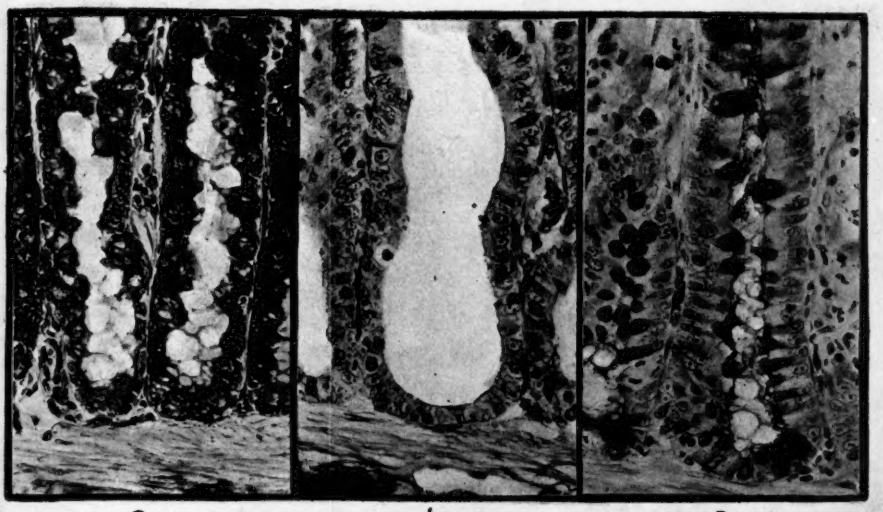


Fig. III

Fig. IV



a b c

Fig. V

d

Fig. V

Fig. VI

THE MECHANISM OF GOBLET-CELL SECRETION IN THE MAMMAL; THE EFFECTS OF CYANIDE.

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In a previous paper (Florey and Webb, 1931) a description has been given of the histological changes involved in the rapid evacuation of goblet-cells. The experiments here detailed had as their object the further analysis of the mechanism by which the mucin was rapidly extruded. When examined in the fresh state much of the mucus present in goblet-cells exists as discrete droplets, though many cells are to be met with containing at least some homogeneous clear "mucin." During intense secretion, caused by the application of dilute mustard oil, the droplets or clear substance are extruded, passing into solution to form intestinal mucus somewhat of the consistency of fresh egg-white. This process seems to be essentially the solution of the intracellular mucin to form a more dilute gelatinous product. Histologically there appears to be no reason for supposing that the mucus after extrusion is of different chemical constitution from that of the intra-cellular product. It might be considered that the act of secretion was the active passing of water through the cell, the mucin being dissolved—or diluted during this passage. It is also possible that the mucin droplets have the physical characteristics of their surfaces so altered as to be more easily soluble, or that an irritant might alter their character so that they imbibe water and are thus extruded. Both processes might occur simultaneously.

The secretion of the mucus might therefore be considered as a physical process, independent of oxygen consumption on the part of the cell. For testing this assumption use has been made of cyanide, which inhibits nearly all cellular oxidation.

EXPERIMENTAL.

Recourse has been had to perfusion of a loop of colon by means of a pump. A Dale-Schuster pump has been used to propel either defibrinated or heparinized cat's blood through a preparation of colon. The actual blood-flow through a small piece of colon is not great, so a large by-pass arrangement was included. Adequate oxygenation was secured by Drinker's apparatus. The bulk of the circulated blood passed through the shunt, so that the blood really circulated several times through the oxygenator for once through the organ, thus ensuring an adequate oxygen uptake. The colon preparation was made as follows: Two loops of string were passed round the colon. The inferior was tied at the

* With a grant from the Medical Research Council.

level of the approximation of the vessels to the gut wall, the vessels being included in the ligature. After evacuation of fæces, the other ligature was tied higher up so that a loop of colon 6 cm. or so long was included. The aorta was tied just below the exit of the inferior mesenteric artery. Cannulæ were inserted into the longitudinal vein and aorta as illustrated (Fig. 1). A preliminary wash through with saline from a syringe was made to avoid trouble from clotting in the relatively small cannulæ. The colon after excision was pinned out on a piece of cork to enable it and the cannulæ to be more easily handled and the cannulæ were then connected to the pump. The glass parts of the pump and perfusion apparatus were immersed in a tank of water kept at

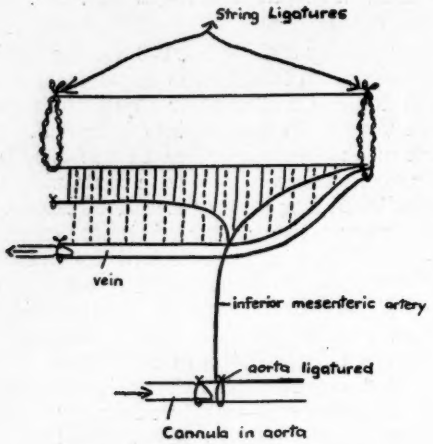

Fig. 1

37° C. At one end of the tank a small metal dish was arranged so that its contained saline was kept at 37° C. by the water-bath. The piece of colon was submerged in this saline during the perfusion. The perfusion pressures varied from 70 mm. to 120 mm. Hg. and, as is commonly the case, the pressure had to be raised during the experiment to keep up an adequate supply of blood. The colon when perfused is this manner continued to use oxygen, as the venous return was always considerably darker than the arterial supply. The length of time of perfusion varied from 3 to 4 hours.

DESCRIPTION OF FIGURES.

Fig. 1.—Diagrammatic representation of the colon preparation ready for perfusion.

Fig. 2.—(*a*) Normal crypts of colon. Cat. Mg. × 130. (*b*) From same animal after 3½ hours' perfusion. Note almost complete absence of goblet-cells. The crypts now lined by cubical and cylindrical epithelium. Compare with Fig. 1*b* of previous paper. Mag. × 130. (*c*) Perfusion of colon with cyanide fluid for 3½ hours. Note presence of goblet-cells even at bases of crypts. Mag. × 130. All sections stained by Heidenhain's iron-alum hæmatoxylin.

(Microphotographs by H. P. Hudson.)

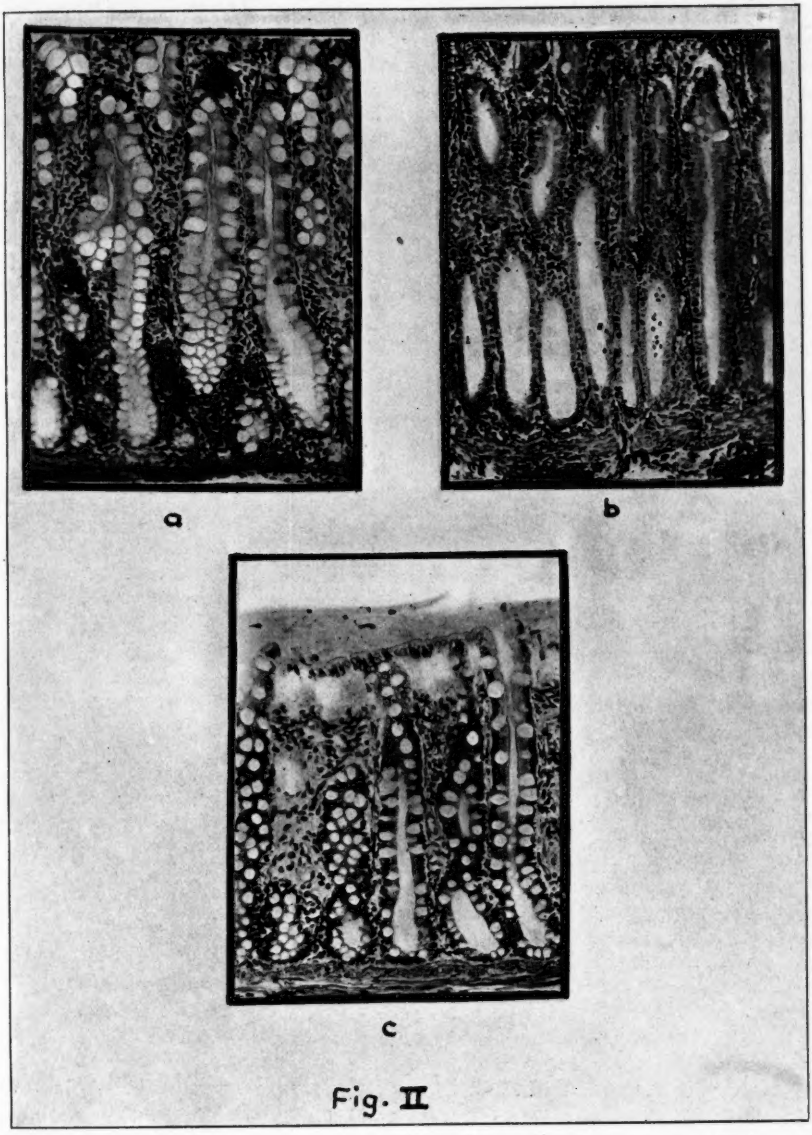

Fig. II

HISTOLOGICAL.

A piece of normal colon was fixed for comparison with pieces of perfused colon. The general procedure was the same as described in the previous paper. In two cats in which the colon was simply perfused for 3½ hours, it was evident that considerable macroscopic mucus secretion had occurred without placing any irritant in the lumen. Histologically the goblet-cells, especially at the bases of the crypts, showed all the signs of rapid secretion previously described. In other experiments mustard oil (8 drops in 25 c.c. olive oil) was injected into the loop by means of a large needle and syringe at the beginning of perfusion. In one experiment (see Fig. 2*b*) there was a condition very similar to that produced in the colon of the intact animal by similar treatment. The goblet-cells at the bases of the crypts had completely disappeared, to be replaced by a cylindrical or cuboidal epithelium. Some goblet-cells were present in the upper portions of the crypts, which also showed epithelial desquamation. The colon providing this histological picture was somewhat œdematous at the end of 4 hours' perfusion, and the contents of the lumen were slightly blood-stained.

In other similar experiments, although the exhaustion was not as complete as that in the case figured, the goblet-cells of the bases were emptied, or were clearly in one of the later stages towards discharge described in the previous paper. It was thus evident that the goblet-cells of the perfused cat's colon were capable of reacting to the stimulus of mustard oil in a manner essentially similar to that of the intact animal. A difference, however, was that no mitoses were noticed in the stimulated perfused colon.

The effect of the addition of cyanide to the circulating fluid was then tried. A normal saline solution containing M/300 HCN was injected into the vessels of the colon loop as soon as possible after the insertion of the arterial cannula to avoid any change depending on oxygen during the manipulations. A mixture as recommended by Starling and Verney (1925) of M/600 to M/300 HCN was mixed with the blood and propelled by the pump. Cyanide solution was added from time to time to ensure an adequate quantity being continually present.

The onset of œdema in the preparations submitted to the action of cyanide was relatively rapid, owing to the increased permeability of the blood-vessels, which tended to become filled with corpuscles and blocked. As exclusion of oxygen was the primary object of the experiment, clearly, corpuscles were unnecessary. The plasma of heparinized blood to which the HCN mixture was added was therefore substituted and perfused through by a simple apparatus using gravity for the production of perfusion pressure. This pressure was kept low—20 to 25 mm. Hg.—and an adequate supply of fluid to the colon was maintained, though even in the absence of corpuscles the flow tended to diminish with the progress of perfusion. Mustard oil (8 drops in 25 c.c. olive oil) was injected into the loop of colon as before and this was kept immersed in saline at 37° C.

In no case was there any emptying of the goblet-cells at all comparable to that in the non-cyanided preparation (Fig. 2*c*). Macroscopically these colons showed a thin watery mucus layer on the epithelial surface of the colon.

Microscopically the following changes were noted; the experiment showing them to the most marked extent is described, though they were present to a lesser degree in others: The depths of the crypts contained mucus which took the muci-carmine stain very intensely. This mucus could be traced as continuous with that in goblet-cells, many of which had wide open mouths. The cells containing the mucin all maintained their goblet appearance, *i. e.* there was no formation of cylindrical or cubical epithelium free from mucin. In some cases, both in the depths of the crypts and towards the surface, the goblet-cells appeared to have disintegrated, their content mingling with that coming from still intact cells, and being in no way distinguishable from the mucin of the latter.

This description applies to the experiment giving the most marked result. Three other similar experiments gave only slight evidence of the egress of mucin.

DISCUSSION.

The following interpretation gives a reasonable explanation of the histological results. It is clear that the cells stimulated in the presence of oxygen do not present an absolutely sharp differentiation from those treated with cyanide.

The secretion of the goblet-cell is essentially a passage into solution of its concentrated mucin content. To effect this the cell has clearly to pass water through itself from the blood-stream to the gut lumen. It is for this active passage of water that the energy supplied by oxygen is necessary. If this mechanism is impaired by cyanide a certain small amount of water passes through the cells, which have probably had their permeability increased by the cyanide in the same way as that of the blood-vessel endothelium. This water carries with it a certain small amount of mucin into solution, the process being a physical one. In the cyanide perfusion experiments the tissues became very œdematous, that is, there was plenty of water available for the cell to add to its mucin content if it had been capable of doing so. This view is strengthened by the observation (previous paper) that complete goblet-cells can become detached from the mucosa without discharging their contents, although suspended in secretion containing plenty of water. They are cut off from their oxygen supply. That the actual solution of the mucin granules is a physical process can be shown by pressing in a mortar and pestle a piece of colon mucosa in a solution of sodium cyanide in which it has previously been soaking to avoid any influence of oxidative processes. The mucin is pressed out of the goblet-cells and the piece of mucosa becomes covered with "egg white" mucus. Histologically a piece of mucosa so treated shows rather collapsed and elongated goblets with a considerable amount of mucus in the lumina of the crypts. This mucus is indistinguishable histologically from that produced by "secretion" except that it stains more readily, being, presumably, more concentrated.

SUMMARY.

Oxygen is necessary for the rapid discharge of goblet cells when stimulated. It is suggested that stimulation (any cause of "inflammation") results in a

stream of water being passed through the cell. This dissolves the mucin, which is passed out through the open mouth of the cell. Oxygen is necessary for the transport of the water. At the same time it is conceivable that the intra-cellular mucin becomes more able to deal with the water supplied by altering its power of imbibition. The present experiments do not give any evidence on this matter.

I am much indebted to Prof. E. B. Verney for advice in the construction of the perfusion apparatus and for the loan of a Dale-Schuster pump.

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THE SIGNIFICANCE OF NATURAL VARIATIONS IN THE STRUCTURE AND CORTICAL LIPOID OF THE MOUSE SUPRARENAL.

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MASUI and Tamura (1924) described a juxtamedullary zone in the suprarenal cortex of young adult nulliparous female mice without counterpart in the adult male cortex. It was termed the " X zone " by Miller (1927), who found that it first became evident in both sexes at 14 days after birth. In males it had disappeared at 38 days; in females, however, it continued to grow, gradually disappearing by a fatty change, so that at 80 to 200 days, glands from males and females were indistinguishable. Miller's results were supported by Preston (1928).

In this paper evidence of a sex difference in the amount of cortical lipoid in the mouse suprarenal is brought forward. The recognition of this natural variation and of the X zone necessitates a new interpretation of findings that have so far been considered abnormal and made the basis of a theory of suprarenal function.

MATERIALS AND METHODS.

The suprarenals of 65 healthy mice of known age and sex were examined. In 4 of them worm-cysts were found in the liver at autopsy. No other gross abnormalities were seen. Only the suprarenals were examined microscopically. All the mice were killed by fracture of the neck. Three fixatives were used: 10 per cent. formol saline; 5 per cent. potassium bichromate followed by 10 per cent. formol saline; osmic vapour. Glands fixed in the first were cut

frozen and stained with Sudan III and hæmalum. The second is that recommended by Ogata and Ogata (1917, 1923) after detailed study of the chrome reaction, and gave excellent results. They found that acid alkali and sublimate interfere with the chrome reaction. Helly's fluid, containing sublimate, is approved by Goldzieher (1929), but in my hands has never given good results. Fixation in osmic vapour was effected by Cramer's technique (1928). Despite removal of the periadrenal fat it often failed, but it is better to fix many glands whole than to bisect a few, since bisection distorts them and produces misleading pictures in the medulla. The stains used were hæmalum and eosin and Mallory's stain for fibrous tissue.

NOMENCLATURE.

The classical division of the suprarenal cortex into three zones is not used in the following descriptions of the mouse suprarenal, since its use would imply the existence of sharper distinctions than those observed. The arcades of cells found just beneath the capsule of the gland correspond to the glomerular zone, but the rest of the cortex, composed of regular cell-columns, corresponds to the fasciculate zone only. Its inner margin adjoins either the fibrous band limiting the medulla in glands without an X zone, or the X zone itself when present. There is sometimes slight irregularity of the inner ends of the cell-columns, but this irregularity is not constant, and therefore it seems unnecessary to denote it by the term "reticular zone." The term "reticularis" is currently used to denote the slight irregularity just described, as by Miller (1927), and also to denote the X zone itself, as by Cramer (1928). The term "X zone" was suggested by Miller in order to distinguish the temporary cortex from the permanent cortex, at whose inner margin the true reticular zone, if it occurs, is to be found. The value of the threefold division is further reduced by the observation that in mice the entire cortex, including the "glomerular zone," sometimes stains homogeneously with Sudan III.

THE NORMAL MOUSE SUPRARENAL.

(A) *In the Male at* 142 *Days* (Fig. 1).

Five glands from 5 animals were studied. The gland is invested by a fibrous capsule. Beneath this the cells are arranged in irregular arcades, merging gradually with the columns of larger cells forming the rest of the cortex. Approximately the outer half of each cell-column is composed of cells whose

EXPLANATION OF PLATE.

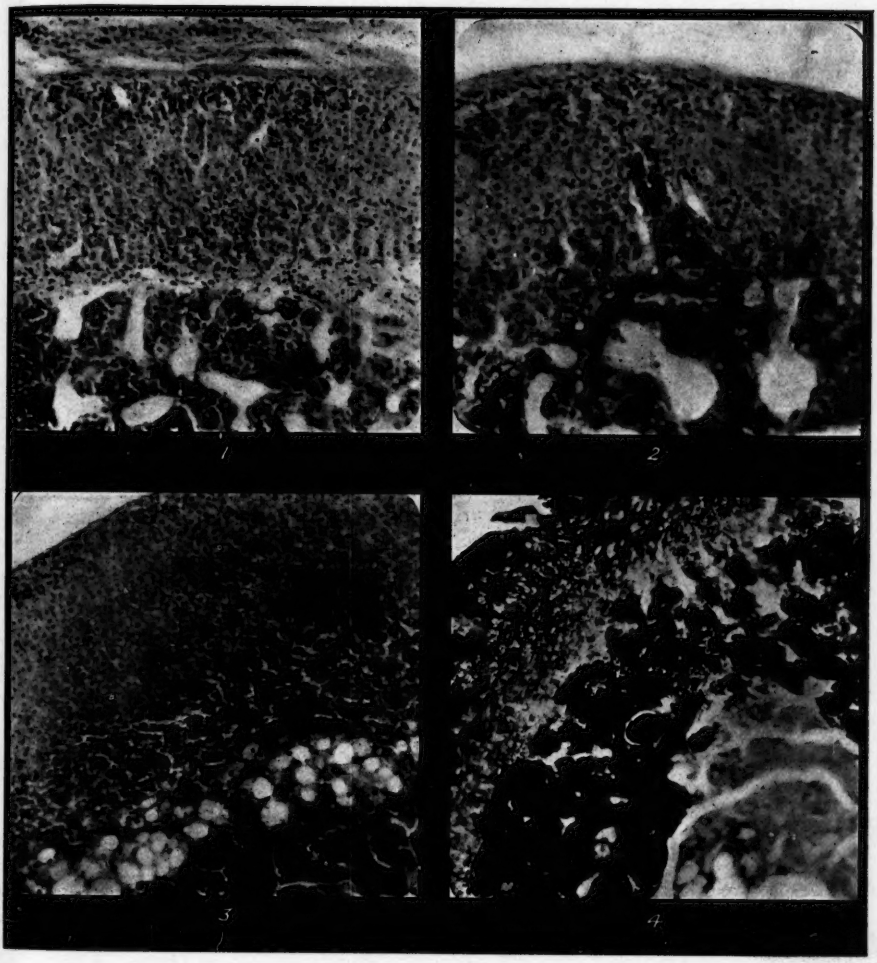
FIG. 1.—Suprarenal of normal male mouse, aged 142 days, showing fibrous band between cortex and medulla, whose edge is even. Stain: H. and E. after chromation. × 150.

FIG. 2.—Suprarenal of normal male mouse, aged 142 days, showing cord of medullary cells in cortex. Stain: H. and E. after chromation. × 200.

FIG. 3.—Suprarenal of normal female mouse, aged 142 days, showing X zone, inner third of which is vacuolized. Edge of medulla uneven, with no fibrous band between it and cortex. Stain: H. and E. after chromation. × 150.

FIG. 4.—Suprarenal of normal female mouse, aged 163 days, showing complete fatty change of X zone. Unstained: Osmic vapour fixation. × 150.

Photographs by H. C. TAYLOR.



Whitehead.

cytoplasm is spongy and stains faintly with eosin. These are cells whence lipoid has been dissolved during preparation of the section. The inner half of each column is composed of cells whose cytoplasm appears homogeneous and stains deeply with eosin. There is no structural discontinuity between the lipoid-rich and the lipoid-free cells of each column. The columns are sometimes slightly irregular at their inner ends.

The medulla has an even edge, and a band of fibrous tissue perforated by blood-vessels separates it from the cortex. Delicate strands of fibrous tissue radiate from the perimedullary band through the cortex to merge at its outer margin with the capsule. Internally, coarser and less regular offshoots form the stroma of the medulla. The cells of the medulla are arranged in compact groups. Their cytoplasm is dark brown after chromation, but the same gland shows variations in tint from one cell-group to another. The cells of the medulla and their nuclei are larger than the cells and nuclei respectively of the adjacent cortex, and this difference makes their distinction easy, even without previous chromation.

In one gland (Fig. 2) a cord of medullary cells was seen in the cortex. The chrome reaction, intense in these, was never seen in a true cortical cell.

(B) *In the Female at* 142 *Days* (Fig. 3).

Five glands from 5 animals were studied. All showed the X zone, its breadth ranging from 0·3 to 0·5 of that of the whole cortex. The permanent cortex, defined as cortex minus X zone, is morphologically identical with the whole cortex of the adult male, and need not be described.

The X zone is composed of irregularly arranged strongly eosinophil cells about half the size of the pale cells of the permanent cortex. The nuclei of the X zone cells are equal in size to the nuclei of the pale cells of the permanent cortex, and stain deeply with hæmalum. The outer limit of the X zone is sharp. There is no fibrous band separating the X zone from the medulla, the cells of the two tissues being in close contact. The appearance differs from that of glands of either sex whence the X zone has disappeared, where a fibrous band intervenes between cortex and medulla.

Three glands showed different phases of a fatty change within the cells of the X zone indicated by unstained vacuoles in paraffin sections, and various stages were seen in the same gland. It was most advanced nearest the medulla. Interdigitation of X zone and medulla was present, but not marked. The average breadth of the permanent cortex was about 0·8 of that of the whole cortex in the males.

(C) *In the Female at* 282 *Days.*

Two of the 5 glands studied showed remnants of the X zone. The other glands were indistinguishable from those of the males, except that the perimedullary fibrous band was broad. The edge of the medulla was even, and its cells sharply separated from those of the cortex. The average breadth of the cortex exceeded 0·9 of that of the cortex in the males.

One gland from each of the 15 mice just described was cut frozen. The sections from the females, aged 142 days, showed sudanophil droplets in the

X zone roughly corresponding in number and distribution with the clear vacuole seen in the paraffin sections of the other gland from the same animal.

Both glands from 30 normal mice were fixed in osmic vapour, and sections of each examined before and after treatment with turpentine, and also stained with hæmalum and eosin after decolourization with hydrogen peroxide. Ten mice were males, aged 164 days, 10 females, aged 163 days, and 10 females aged 305 days. The results confirmed those detailed above. The male glands showed no X zone, all the glands from females aged 163 days did, and 4 of the glands from females aged 305 days showed traces. Fatty change in the X zone was seen in 8 out of 19 glands from the females aged 163 days. One gland showed complete replacement of the X zone by black-stained globules, which formed a band of roughly constant width encircling the medulla (Fig. 4).

A SEX DIFFERENCE IN CORTICAL LIPOID.

The term "lipoid band" is used to denote that part of the permanent cortex whose lipoid-rich cells stain with Sudan III or osmic acid. In the mouse suprarenal this band is usually of uniform width and density. It is to be distinguished from the fat of the involuting X zone which is, of course, excluded by definition from the measurements.

The breadth of the lipoid band was measured with a micrometer in the Sudan III stained sections, and in the sections from glands fixed in osmic vapour after treatment with turpentine. This removes the lipoid, leaving clear spaces easily distinguished from the cells that did not contain lipoid, which remain dark brown or black. A systematic error is possible owing to the personal factor in determining the limits of the lipoid band.

TABLE I.—*Ratios of Cortical Lipoid Band* (L.) *to Total Cortex* (T.C.) *and to Permanent Cortex* (P.C.) *in Normal Mouse Suprarenal.* T.C. *and* P.C. *are each taken as* 10 *units.*

Sex.	Age, days.	Glands studied.	L./T.C.			L./P.C.		
			Average.	Maximum.	Minimum.	Average.	Maximum.	Minimum.
♂	142	5	6	7	6	6	7	6
♂	164	17	5	7	2	5	7	2
♀	142	4	5	6	4	9	10	8
♀	163	19	6	8	3	9	9	7
♀	282	4	8	9	7	10	10	9
♀	305	17	7	9	6	9	9	6

The results are shown in Table I. The measurements are tabulated in two blocks. The left block shows the ratio of the breadth of the lipoid band to the breadth of the total cortex, measured from the outer margin of the gland-capsule to the outer margin of the medulla. The right block shows the ratio of lipoid to permanent cortex, which is the difference between the width of the total cortex and that of the X zone. The total cortex (T.C.) and permanent cortex (P.C.) are taken as 10 units, to avoid a cipher and decimal point before the figure in each entry.

The first column of each block gives the average breadth of the lipoid band in the number of glands stated. The other two columns give the extreme individual measurements. Figures in the odd lines were obtained from Sudan III stained frozen sections, figures in the even lines from section of osmic vapour fixed glands.

Table I brings out two interesting facts: A close correspondence between the measurements of the frozen sections and those of the paraffin sections, and a difference in the amount of lipoid in males and females of the same age. The first four lines in the left block do not suggest that there is any constant difference in the amounts of lipoid in the cortex of the two sexes. This is the impression that is gained from the casual inspection of sections. The corresponding entries in the right block show that when the width of the X zone is subtracted so that the amount of lipoid in the morphologically equivalent tissues of both sexes is measured, the female gland contains a relatively greater amount of lipoid than the male gland. The measurements in the 5th and 6th lines indicate that lipoid occupies nearly the whole of the cortex in females from whose suprarenals the X zone has almost or quite disappeared. Whether age changes occur in the amount of lipoid in the male cortex is at present under investigation.

In female glands lipoid occupies the whole of the permanent cortex with the usual but not invariable exception of the cells that lie just beneath the capsule of the gland. This rim of cells is usually lipoid-free in the male glands also, but the lipoid in the male gland is absent from the inner part of the cortex. This lipoid-free part of the cortex is morphologically different from the X zone, and the difference between them is obvious in sections of glands fixed in osmic vapour stained after decolourization with hydrogen peroxide.

CORTICAL LIPOID IN THE ACTIVE GLAND.

The suprarenals were stimulated in 20 mice, either by exposure to cold and wet, or by the subcutaneous injection of 0·15 c.c. of 1 : 10,000 adrenalin. After exposure to cold the mice were killed at once, and 15 to 20 minutes after injection of adrenalin. All glands were fixed by Cramer's method.

The vessels of the medulla were loaded with dark granules, the picture duplicating that described by Cramer (1928). No gland was found exhausted.

TABLE II.—*Ratios of Cortical Lipoid Band* (L.) *to Total Cortex* (T.C.) *and to Permanent Cortex* (P.C.) *in the Active Mouse Suprarenal.* T.C. *and* P.C. *are each taken as* 10 *units.*

Sex.	Age, days.	Glands studied.	L./T.C.			L./P.C.		
			Average.	Maximum.	Minimum.	Average.	Maximum.	Minimum.
♂	142	8	5	7	4	5	7	4
♂	143	7	6	7	4	6	7	4
♀	142	3	7	7	7	9	9	9
♀	143	4	7	8	6	9	9	9
♀	163	6	4	6	2	7	10	5
♀	303	5	6	8	2	6	8	2

The cortical lipoid was measured with the results shown in Table II. The glands listed in the 2nd and 4th lines were from mice that received adrenalin. All the others were from mice exposed to cold.

The lipoid shows no change from the normal in the glands from males, nor in those from females aged 142 days (cold and wet for 2 hours), or 143 days (adrenalin). In the females aged 163 and 303 days, however, there is evidence of a real decrease in lipoid as compared with the normal. These mice suffered cold and wet for 4 hours. Where differences in the figures in the two blocks occur, they are due to the X zone as explained above. The constant difference between the relative amount of lipoid in the male and female cortex from mice of the same ages is as clear in Table II as in Table I.

DISCUSSION.

The results confirm those of Miller (1927) on the structure and involution of the X zone, but do not support Cramer's impression that when the medulla has been stimulated there is a centripetal movement of lipoid. The change, when it occurred, was a diminution. Since, in his book (1928), Cramer states that " the adrenals of all mammals which have been investigated so far present at birth the complete appearance of the fully developed adult adrenal " (p. 138), it is evident that the existence of the X zone in mice as a normal phenomenon escaped his notice.

Describing the effect of adrenalin (p. 96) Cramer refers to his Plate I as the normal. It represents a gland without an X zone. As the effect of adrenalin he describes a gland with an X zone. This is the histological basis for his theory of suprarenal self-control. His remark (p. 97) that " this change is induced more readily in female than in male animals," can be explained by the longer persistence of the X zone in females. The observations reported in this paper demonstrate that the appearances described by Cramer are not the result of stimulation of the gland, but are natural sex differences. The structural details are the same in the glands from mice of the same age and sex, whether stimulated or not. The only difference is that the changes within the cells and blood-vessels of the medulla seen after stimulation are absent from the resting gland. Cramer's view would necessitate the disappearance within 15 to 20 minutes after injection of adrenalin of a band of fibrous tissue and the subsequent inosculation of cortical and medullary cells.

The adrenalin-free peripheral zone that Cramer regards as evidence of self-control is stated (p. 98) to be especially distinct after prolonged thyroid feeding, and Cramer believes that he proved conclusively that this zone belongs to the medulla by stopping the thyroid feeding and noting the appearance of the gland three days later. Actually no conclusion is possible since no evidence is adduced of the state of the gland at the moment thyroid feeding was stopped. His Plate 26, alleged to illustrate his contention, shows a normal medulla.

A mouse with spontaneous exophthalmic goitre is stated to show " very profound pathological changes," which are depicted in his Plates 29–31. They illustrate a condition indistinguishable from complete vacuolization of the X zone as shown in Fig. 4 of the present paper, which disproves Cramer's statement beneath his Plate 29 that " this appearance (of cells filled with large globules of fat or lipoid) is never found in the adrenals of normal mice."

SUMMARY.

Lipoid is relatively more abundant in the suprarenal cortex of female than of male mice, aged about 150 days. The changes seen after stimulation of the gland were activity of the medulla and sometimes a decrease in cortical lipoid. The recognition of natural sex differences in the mouse suprarenal removes the histological basis for Cramer's theory of self-control. Cramer's interpretations cannot be accepted since they were made on the assumption that natural variations do not occur in the mouse suprarenal.

I am indebted to Prof. John Shaw Dunn for his helpful criticism.

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GLANDULAR FEVER II.—THE PROTOZOAL NATURE OF THE EXPERIMENTAL DISEASE.

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A PREVIOUS paper (Bland, 1930) described a disease of rabbits that followed the inoculation of blood from a human case of glandular fever and could be transferred indefinitely in serial passage by inoculation of the citrated blood. No suggestion was made as to the kind of infective agent causing this disease, but further study has made it plain that the organism responsible is a protozoon of the genus *Toxoplasma*, Nicolle and Manceaux, 1909, closely resembling the *T. cuniculi*, a natural parasite of the rabbit, but differing from it in at least one important way: its ability to infect monkeys.

When it became plain that this protozoon was probably the cause of the disease in rabbits, a strain of true *T. cuniculi* was got from the Pasteur Institute in Paris, for which my thanks are due to Dr. C. Levaditi, and a number of experiments were made to compare the two. It is the purpose of this paper

to describe this investigation and to offer the evidence for thinking that the protozoon is the cause of the rabbit disease and may perhaps be responsible for human glandular fever.

EXPERIMENTAL.

Two strains of protozoa derived from cases of glandular fever have been studied. The first, which will be called the GF I strain, appeared in animals inoculated with the blood of a case occurring in June, 1930. The second, which will be called the GF II strain, similarly appeared in animals inoculated with the blood of a second case occurring in November, 1930. The two strains appeared to be identical in every way in which they were studied, but the GF II strain was unfortunately lost after seventeen passages and a complete comparison was not effected. A third strain, one of true *T. cuniculi*, has been studied. This is one of those found by Levaditi and his collaborators and described by them (Levaditi, Sanchis-Bayarri, Lépine and Schoen, 1929). It will be known here as the T strain. None of the strains is cultivable and therefore throughout this paper, where the term inoculation with a strain is used, it must be understood to mean inoculation of material, usually blood, from an animal showing typical symptoms of the disease. In most cases this material cannot be shown to contain toxoplasms, and in fact during this work they have never once been demonstrated in infective blood.

Morphology.

The protozoa of the GF strains are morphologically the same as those of the T strain, and correspond exactly with the text-book descriptions of the members of the genus *Toxoplasma*. For such descriptions and for a general review of the subject the reader is referred to the text-book of Wenyon (1926) and the monograph of Chatton and Blanc (1918).

Occurrence of Protozoa in Infected Animals.

The protozoa of all three strains are found in almost all animals inoculated with infective material. Those of the GF I strain were not detected in the original rabbit inoculated from the human case and did not appear in rabbits until the fourth generation, but they were found in the primary guinea-pigs inoculated from this case. The GF II protozoa, were found in two primary rabbits inoculated from the second case, both of which gave typical clinical reactions, but were not found in one rabbit which did not react or in any of the three primary guinea-pigs, although one of those gave a typical clinical reaction. It is sometimes impossible to detect protozoa, in spite of very careful search, in a few of the animals inoculated with any of the three strains although they show typical clinical reactions and post-mortem appearances. This is an experience that has been met by others who have studied toxoplasmosis. Organisms resembling the GF protozoa have only once been found by me in a large number of control rabbits that were inoculated with rabbit blood or with human blood from normal or febrile people. In this rabbit, which was inoculated with rabbit blood to which it did not react in any abnormal way, a few

protozoa resembling toxoplasmata were found in the spleen after a prolonged search. None could be found in animals inoculated from this rabbit, all of whom remained normal.

Comparison of the Effects of Inoculation with the GF Strains and with the T Strain.

The effects of inoculation of infective material from any of the three strains are identical and are those described in the previous paper (Bland, 1930). Typical reactions as far as the temperature and blood picture are concerned are shown in Figs. 1 and 2. The pathological changes, both macroscopic and microscopic, are identical whether produced by GF strains or by the T strain.

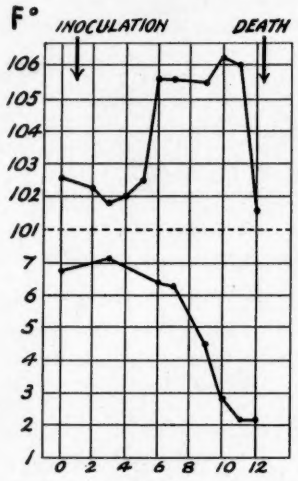

Fig. 1.

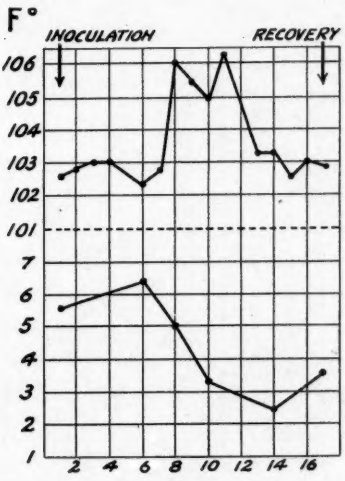

Fig. 2.

Fig. 1.—Rabbit CL 348, Strain GF I, 5th generation. The upper half of the figure gives the temperature chart, the lower the erythrocytes. Abscissæ refer to days after inoculation, ordinates to degrees Fahrenheit, with the thermometer 6·5 cm. in the rectum, and to millions of erythrocytes per cmm. All other charts in this paper are drawn up on the same plan.

Fig. 2.—Rabbit CL 557, Strain T., 5th generation.

They consist of enlargement of the lymph-glands, liver and spleen, areas of focal necrosis in the latter organs and a pneumonia of unusual type with mononuclear exudate and infiltration of the alveolar walls. There is an important difference between the virulence of the strains. The GF strains are very lethal and it is extremely rare for rabbits to recover from them: the animals usually die in about seven to ten days when the temperature has fallen. The T strain on the other hand only rarely kills: at the end of the febrile period the temperature falls to normal and the rabbit's condition rapidly improves.

All three strains are infective for guinea-pigs and mice.

Infection of Monkeys with the GF I Strain.

All previous attempts to infect monkeys with toxoplasmata have failed (Chatton and Blanc, 1917; Levaditi and collaborators, 1929), and on the two occasions when monkeys were inoculated with infective T strain blood they remained entirely normal. On the other hand, on three occasions out of four when monkeys were inoculated with infective GF I strain blood from rabbits they have responded in a very striking way which seems worthy of record in some detail.

Monkey I.—Macacus rhesus inoculated November 6th, 1930, intraperitoneally with 5 c.c. of citrated rabbit blood of strain GF I, 16th generation. Fig. 3 and Table I show that this monkey responded after an incubation period of ten days with pyrexia, an anæmia and a very big increase in the large lymphocytes of the blood, many of which were of the primitive or pathological type with deeply basophilic cytoplasm. On the nineteenth day after inoculation and the eighth of fever, packets of swollen tender glands could be felt in both axillæ and both groins where none were felt before. The monkey was killed for examination two days after the fall of temperature; it had never seemed very ill and would probably have made a complete recovery. A rabbit inoculated with the monkey's blood contracted a typical attack of the disease and protozoa were found in its organs.

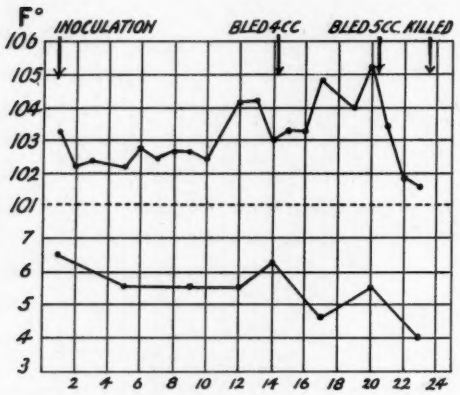

FIG. 3.—Monkey I, Strain GF I, 17th generation.

TABLE I.—*Monkey I, Leucocyte Counts.*

Days after inoculation.	Total leucocytes per c.mm.	Polymorphonuclears per cent.		Lymphocytes per cent.		Monocytes per cent.
		Neutrophils.	Oxyphils.	Small.	Large and primitive.	
12	..	63	0	22·5	10	4·5
17	8,300	26	1·5	26·5	43	3
20	11,900	30·5	1·5	17·5	46	4·5
23	13,300	13	0·5	19·5	61·5	5·5

Post-mortem in this monkey were found a general enlargement of the *lymphatic glands;* those from the axilla were largest and were soft in texture and purple in colour. The *spleen* was moderately enlarged and weighed 6·2 gm. It was a reddish purple colour with smooth peritoneal surface. On cutting the texture was firm and there was no bulging of the edges; the cut

surface was a bright red with very numerous grey Malpighian bodies the size of a full stop. There were no areas of focal necrosis. The *liver* weighed 85 gm. and was normal in colour and appearance ; the lobular pattern was not marked and there were no focal necroses. The *lungs* were a greyish purple colour mottled with paler areas which stood above the general surface ; both seemed rather collapsed, but were aerated and floated in water. No fluid or mucus could be expressed from the cut surface. The *bone-marrow* of the femur was a deep red throughout the shaft of the bone and was very soft and friable. The rest of the post-mortem was negative.

The histology of this monkey's organs differs in an important way from the picture in the rabbit. The massive focal necroses in the spleen, liver, lungs and lymph-glands which are such a feature of the disease in the latter animal are entirely absent. In the *spleen* there is a hyperplasia of the germinal centres of the Malpighian bodies, some engorgement of the pulp and a diffuse necrosis

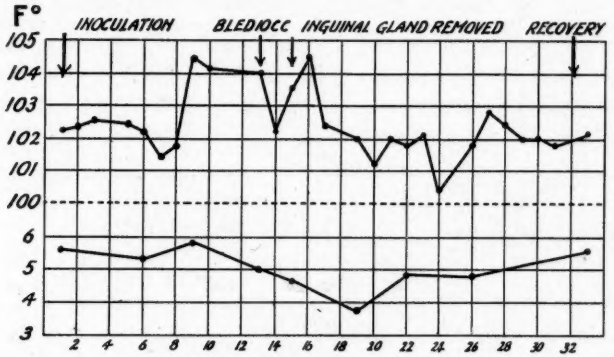

FIG. 4.—Monkey IV, Strain GF I, 41st generation.

of many individual cells both of the pulp and of the lymphoid tissue, but these are nowhere collected together into foci. The *liver* shows a few minute foci of necrosis having no special arrangement within the lobule. These are formed of at most half-a-dozen necrotic liver-cells surrounded by small round-cells and reticulo-endothelium. The liver-cells are foamy and granular suggesting advanced parenchymatous degeneration and early fatty change. The *lungs* are on the whole very similar to those of rabbits with this disease but no areas of focal necrosis are present as they often are in that animal. There is a patchy pneumonia of a peculiar type in which the alveolar walls are greatly thickened by the presence in them of large pale mononuclear cells. This thickening is so great as sometimes to obliterate the alveolar cavity. Although a certain amount of this consolidation is due to catarrh of the alveolar cells this is slight and very little exudate is formed. The bronchi and bronchioles are relatively little affected, even those in the midst of a consolidated patch have an intact epithelium and hold no exudate. Their walls, however, are sometimes infiltrated with round-cells. The *lymph-glands* present a most interesting picture : there is much change in them and this is essentially of the type found in human

glandular fever, namely a great over-growth of the reticulum cells and of the cells of the sinuses. These changes are not so great as in human glands and although the alterations are in neither case entirely specific the resemblance is striking. No protozoa could be demonstrated in smears or sections of the organs of this monkey in spite of a very careful search.

Monkey IV.—Macacus rhesus inoculated intraperitoneally April 13th, 1931, with 5 c.c. of citrated infective rabbit blood of strain T, 7th generation, showed no significant reaction and was inoculated intraperitoneally May 14th, 1931, with 7 c.c. of citrated infective rabbit blood of strain GF I, 40th generation.

TABLE II.—*Monkey IV, Leucocyte Counts.*

Days from inoculation.	Total leucocytes per c.mm.	Polymorphonuclears per cent.		Lymphocytes per cent.			Monocytes per cent.
		Neutrophils.	Oxyphils.	Small.	Large.	Primitive.	
4 before.	11,000	55	1·5	36·5	3·5	0	3·5
9 after	7,200	65	0·5	27·5	3·5	0	3·5
13 ,,	5,400	52	1·5	26	11·5	4	5
15 ,,	7,600	25	0·5	56	4·5	9	5
19 ,,	24,000	19	1	43·5	5·5	25·5	5·5
22 ,,	12,600	21·5	2	45	15·5	13	3
26 ,,	9,000	23·5	1·5	38·5	21	10·5	5
33 ,,	12,200	43·5	0	36·5	10	4	6

To this it responded after an incubation period of seven days with a pyrexial illness of the same type as that given by Monkey I. The chief points of this which are brought out by Table II and Fig. 4 are a pyrexia of eight days' duration followed by an anæmia and a leucocytosis in which the total number of lymphocytes was much increased, while more than a third of them were of the primitive or pathological type. Coincident with the fever there was a general swelling of the lymph-glands. A group of these excised from the groin on the seventh day of fever showed the same histological picture as those of monkey I, and contained no toxoplasms. Monkey IV was allowed to recover and no examination of its organs was done. Passage of this monkey's blood to a rabbit gave a typical reaction, and to a monkey, after the blood had been kept five days, to a somewhat doubtful one.

Monkey V.—Macacus rhesus inoculated July 1st, 1931, with 7 c.c. of blood of Monkey IV which had been kept in the refrigerator five days. There was a short period of irregular temperature, mostly subnormal, with a rise to 103·8° and 103·2° F. on the eighth and ninth days after inoculation. The temperature then settled to the normal level of 102°. On the thirty-sixth, thirty-seventh and thirty-eighth days after inoculation there was another brief period of low pyrexia. Neither of these febrile periods was accompanied by any significant blood changes. On the forty-fourth day the monkey was given a second inoculation of 7 c.c. of infective rabbit blood of strain GF I, 52nd generation. This was followed by an incubation period of normal temperature for five days and a four-day pyrexial period with temperatures of 104·4°, 104°, 103·8° and 104° F. The temperature then became normal and the monkey

made a good recovery. The blood-picture during and for some days after this febrile period showed essentially the same features as in Monkey I and Monkey IV. Generalized enlargement of the lymph-glands appeared on the last day of fever and persisted for several days. A gland was removed from the axilla for histology and showed the same changes as the glands of Monkey I. No toxoplasmata were found. A rabbit inoculated with this monkey's blood contracted a typical attack of the disease.

Monkey VI.—Macacus rhesus inoculated with 7 c.c. of infective rabbit blood strain T, 13th generation, showed no significant blood or temperature alterations. Forty-four days later it was reinoculated with 7 c.c. infective rabbit blood of strain GF I, 51st generation. To this it also failed to respond.

Cross-Immunity Experiments Between the Three Strains.

A number of experiments were done to see if animals immune to the T strain would be still susceptible to the GF I strain. Five rabbits were inoculated with infective T strain blood ; all responded with typical reactions from which they recovered. Seven to fourteen days later they were given a second inoculation of infective T strain blood. To this they showed no response. Seven to ten days later they were given a third inoculation, this time of infective GF I strain blood, and again remained unaffected. The original attack of T strain infection appears to have conveyed immunity from subsequent inoculation with either T strain or GF I strain.

Owing to the difficulty of getting recovered GF I strain rabbits the converse experiment was not tried. Such an experiment was done however with strain GF II. A rabbit was inoculated with infective blood of this strain together with an immune serum prepared against strain GF I. The animal had a typical but very much prolonged illness and its temperature did not settle to normal till eight weeks later. Ten weeks after the primary inoculation it was given a second dose of GF II strain blood to which it failed to respond. Three weeks later an inoculaton of T strain blood was given and again there was no response.

These experiments suggest an immunological identity between the T strain of rabbit toxoplasm and the two strains derived from rabbits inoculated with human glandular fever blood. It is true that the case of monkey IV, described in the preceding section, appears to contradict this, but in reality it does not do so. The original inoculation of T strain blood in this case produced no infection and it is probable that without such infection the amount of antigen given in a single inoculation would be too small to arouse sufficient antibodies to protect against the subsequent inoculation of GF I strain blood.

DISCUSSION.

Discussion of the results just described may be directed towards three points. (1) The ætiological relationship of the protozoa of the GF strains to the rabbit disease. (2) The differences between both these strains and one of true *T. cuniculi*—the T strain. (3) The possible ætiological relationship of the GF strains to human glandular fever.

(1) All the evidence suggests that the protozoa of the GF strains are the actual cause of the rabbit disease they are associated with. The disease itself corresponds in every way but its virulence with that caused by the T strain of *T. cuniculi* and resembles the descriptions of toxoplasmosis that are to be found in the literature. The protozoa are to be found in the majority of animals suffering from the disease, and though they are sometimes absent and infection can frequently be produced with material in which protozoa cannot be demonstrated this is not of much significance, for precisely the same results have been obtained with the T strain and are described by other workers on toxoplasmosis. Finally the protozoa of the GF strains resemble other toxoplasmata so closely that they must be regarded as members of this genus. They behave exactly like the T strain in most essential ways and are immunologically identical with it.

(2) The GF protozoa differ from the T strain very greatly as to their virulence for rabbits and the GF I strain is capable of infecting monkeys. It is not known whether the GF II protozoa also had this power, for the strain was lost after seventeen passages and the experiment was not tried. This infection of monkeys is of great interest from two points of view : firstly, because it is the only recorded instance of a disease having been produced in these animals with any strain of toxoplasm, and secondly, because of the resemblance of the illness to human glandular fever.

(3) This resemblance between the natural and experimental diseases constitutes the strongest single piece of evidence for considering the GF protozoa the cause of glandular fever in man. The cardinal symptoms of the human disease and the chief points in diagnosis are : (1) pyrexia ; (2) generalized swelling of the lymphatic glands ; (3) alteration of the blood-picture in a specific way, especially an increase in the total number of lymphocytes many of which are of the primitive or pathological type. All these signs were unmistakably present in the monkeys infected and indeed a closer correspondence between the natural and the experimental disease could hardly be expected or required. At the same time it must be borne in mind that the GF protozoa have been obtained from only two cases of glandular fever, and although a number of control inoculations much greater than those with glandular fever material have never produced a disease of any kind, there is a natural reluctance to implicate as the cause of a human disease protozoa of a genus members of which are known to be a natural parasite of the rabbit. In such a case unimpeachable evidence is required and this waits upon the obtaining of more cases of glandular fever for study. Their present rarity seems likely to postpone for some time the final solution of this problem.

SUMMARY AND CONCLUSIONS.

1. The rabbit diseases, previously called "experimental glandular fever," which followed the inoculation of blood from two cases of human glandular fever is caused by protozoa of the genus *Toxoplasma*.

2. These protozoa closely resemble the *Toxoplasma cuniculi* and are immunologically identical with it. The disease they cause is indistinguishable from that produced by a strain of *T. cuniculi*.

3. The protozoa differ from ordinary *T. cuniculi* in their greater virulence for rabbits and in their power to infect monkeys in which animal they produce a disease very like human glandular fever.

4. No similar disease has been produced in rabbits of the same stock by control inoculations with normal rabbit or normal human blood or with blood from febrile people.

5. The evidence suggests that human glandular fever may be caused by the protozoa described, but this requires confirmation.

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HUMAN SKIN-TOLERANCE TO DIPHTHERIA TOXIN, AND IMMUNIZABILITY.

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THE experiment described below was done with a view to devising a practical quantitative Schick test. Three batches of new entrants to Greenwich Hospital School were all given two intracutaneous and simultaneous injections of Schick toxin: (*a*) the usual Schick dose of approximately 0·02 m.l.d. diphtheria toxin in 0·2 c.c. of diluent, and (*b*) a quarter Schick dose (0·005 m.l.d.) also in 0·2 c.c. of diluent. The reactions were recorded on the seventh day after insertion of the toxin. Pseudo-reactions excluded, there were 90 boys who reacted to the full dose; of these 48 also reacted to the quarter dose. The latter will be termed "full-positives." The remaining 42, who failed to react to the weaker dose, will be called "semi-positives." These 90 boys were then given two doses of a diphtheria prophylactic with a fortnight's interval between them. They were re-Schick tested one month and three months after the first prophylactic inoculation. As shown in the Table, by this means the "full-" and "semi-positives" were further subdivided into three classes of immunizability—"rapid," "medium" and "slow."

Now Glenny and Waddington (1929) have shown that in guinea-pigs the tolerance of the skin to diphtheria toxin is directly proportional to the titre of antitoxin in the blood. Moreover, in both man and experimental animals,

TABLE.—*Immunizability and Intensity of Schick Reaction.*

Type of Schick reaction.	Number in group.	Schick-immunity acquired. Within one month. "Rapid." Number.	Per cent.	Between two and three. "Medium." Number.	Per cent.	In over three months. "Slow." Number.	Per cent.
"Semi-positive"	42	16	$38 \pm 5{\cdot}0$	12	$29 \pm 3{\cdot}9$	14	$33 \pm 3{\cdot}5$
"Full-positive"	48	12	$25 \pm 4{\cdot}1$	25	$52 \pm 3{\cdot}5$	11	$23 \pm 4{\cdot}3$
Total	90	28	$31 \pm 3{\cdot}3$	37	$41 \pm 3{\cdot}4$	25	$28 \pm 3{\cdot}1$

it is well established that, if a diphtheria antigen is given to Schick positive reactors who have detectable traces of antitoxin in their blood-sera, a large rise in the titre to above Schick level promptly follows, *i. e.* a Schick negative reaction is rapidly acquired. In view of these findings it was confidently expected that all the "semi-positives" would be found in the "rapid" immunizability group, or at least that none would be found in the "slow" group. The Table shows how badly this prediction was fulfilled, and that, for the practical administrative purpose of picking out beforehand those subjects who could with certainty be actively immunized with only one or two doses of prophylactic, a quantitative Schick test is useless.

The result of this experiment is nevertheless of academic interest; because although, in accordance with expectation, "rapid" immunizability was more frequent among the "semi-positives," yet, contrary to expectation, "slow" immunizability was also more frequent among "semi-" than "full-positives." In the "semi-positive" group the "medium" sub-group was the smallest, both the "rapid" and "slow" immunizability groups having been increased at its expense. The numbers, though small, are suggestive that the peculiar frequency distribution of immunizability among the "semi-positives," as contrasted with the "full-positives," more than half of which had "medium" immunizability, was the result of "semi-positiveness" representing two distinct characters:

(*a*) Partial acquired immunity, *i. e.* the subject has diphtheria antitoxin in the blood below the Schick test level (less than 0·03 unit of antitoxin per c.c.). This would account for the high frequency of "rapid" immunizability.

(*b*) Partial skin "anergy" to diphtheria toxin, *i. e.* the subject need have acquired no circulatory antitoxin, but has a genetic natural skin-tolerance to toxin above the average. This would explain the high frequency of "slow" immunizability among "semi-positive" Schick reactors.

DISCUSSION.

The extreme case of specific anergy to diphtheria toxin would give a negative Schick reaction when no diphtheria antitoxin could be detected in the blood. Kellog (1926–7) has reported several such cases; they are, however, undoubtedly too rare in children and adults seriously to effect administrative preventive work. Nevertheless Freidberger and Heim (1929) believe that the negative Schick tests of infants are due entirely to natural skin insensitiveness,

and have nothing to do with diphtheria antitoxin. This extreme view is untenable, but it is true the intensity of the positive Schick reaction does vary in different subjects who may have no detectable antitoxin in their blood. In conformity with Freidberger and Heim's generalization that the sensitiveness of the skin to all irritants is less in infants than in children or adults, negative Schick tests in the absense of circulatory antitoxin may be relatively common in infancy. v. Groer (1929) who severely criticizes Freidberger and Heim's speculations on the Schick reaction, admits that 15 per cent. of newborn infants, in whose chord blood no antitoxin was detected, gave negative reactions to the ordinary Schick dose of diphtheria toxin, but such Schick negative infants would react to larger doses of toxin, which had no effect on infants who had diphtheria antitoxin in their chord blood. The antithesis to these examples of natural insensitiveness, or anergy to diphtheria toxin, is a natural hypersensitiveness, or allergy, which is represented by the rare type of Schick reaction which vesiculates and produces a shallow ulcer of the skin. (This reaction has no relationship whatever with the pseudo- or protein Schick reaction). Finally natural anergy to diphtheria toxin has an important practical side, as is illustrated by the experience of Hercus and his collaborators (1929). A batch of children in a rural district of New Zealand were Schick tested, and the positive reactors actively immunized. Subsequently (by re-testing) it was found that the Schick reagent had lost potency to such an extent that it only gave half the number of positive reactions as were given by a fully potent Schick reagent. It was estimated that about two-thirds of the negative reactions given by the deteriorated Schick toxin would have been positive with the proper reagent. On the theory that these " semi-positive " New Zealanders were partially immune, it was decided not to inoculate them with a diphtheria prophylactic. About six months after these events, diphtheria again broke out in the locality and proved this theory false ; because the incidence of diphtheria was higher among these " semi-positives " than among a control group of children who had not been tested or inoculated. Thus a partial and " natural " insensitiveness of the skin to diphtheria toxin, *in the absence of acquired antitoxin*, does not appear to imply any special resistance to clinical diphtheria.

SUMMARY.

It was found that a group of boys, who had a greater skin-tolerance to diphtheria toxin than the average Schick susceptible, were, as a group, no more easy to immunize actively than the average. That is to say, the intensity of the Schick reaction in man is not always inversely correlated with immunizability as one would expect from experiments on guinea-pigs.

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THE EFFECT OF REPEATED CALF PASSAGE ON THE IMMUNIZING POWER OF VACCINE LYMPH.

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IN a former paper it was shown (Clearkin, 1928) that the activity of a variolous strain of vaccinia maintained by passage through monkey and calf alternately, decreased if passaged through six calves in succession. The experiments described in the present paper were designed to ascertain if this diminished activity was accompanied by any alteration of antigenic power.

Vaccine lymph used in the experiments was collected after growth for 120 hours on the skin of the calf, the pulp mixed with four times its weight of a 50 per cent. mixture of glycerine and distilled water, and titrated four days after collection. Titration was performed by the insertion of 0·1 c.c. quantities of various dilutions intradermally in the shaved skin of a monkey's abdomen. A control lymph maintained by alternate calf-monkey passage was used in each experiment. This was collected at the same time as the experimental batch and treated similarly after collection. The immunizing tests were performed by the intradermal insertion of the required number of minimal infective doses (M.I.D.), and the resulting immunity tested fourteen days later by the intradermal insertion of various dilutions of an active vaccine, a non-immune monkey similarly treated serving as a control.

In the tables the following symbols are used:

+ = a "take"	O = failure to "take."
− = no insertion made	P = papule.
S = slight	V.S. = very slight.

First experiment.—Vaccine lymph obtained after twenty-three successive passages was titrated and gave the following result:

Dilution.	Monkey. 190. 23rd calf passage.	191. Alternate calf-monkey passage.
1/120,000	+	++
1/140,000	O	++
1/180,000	+	++
1/200,000	O	+
1/300,000	O	+

The result of titration of No. 190 being unsatisfactory, both were re-titrated fourteen days later with the following result:

Dilution.	Monkey. 194. 23rd calf passage.	195. Alternate calf-monkey passage.
1/120,000	+	++
1/140,000	+	++
1/180,000	S +	++
1/200,000	O	++
1/300,000	O	+

The lymph obtained after twenty-three successive calf passages was much less active than that obtained after alternate calf-monkey passages.

The immunizing power of 100 M.I.D. and 1000 M.I.D. of each was next tested.

Monkey No. 196 received 100 M.I.D. of vaccine lymph, 23rd passage.
,, 197 ,, 1000 ,, ,, ,, ,,
,, 198 ,, 100 ,, ,, ,, alternate passage.
,, 199 ,, 1000 ,, ,, ,, ,,

Fourteen days later their immunity was tested. The result is shown in Table I:

TABLE I.

Dilution.	Monkey. 196.	197.	198.	199.	Non-immune control.
1/5	P	P	P	P	—
1/10	P	P	P	P	—
1/100	P	P	O	O	—
1/1000	O	O	O	O	—
1/100,000	O	O	O	O	++
1/120,000	—	—	—	—	+
1/180,000	—	—	—	—	+
1/200,000	—	—	—	—	S +

The papule appeared on the immunized monkeys on the first or second day and vanished on the third or fourth day.

In this experiment there is no appreciable difference between the immunity conferred by 100 M.I.D. of vaccine lymph obtained after propagation of the virus through twenty-three consecutive calves and that obtained by propagation in the calf and monkey alternately.

Second experiment.—The calf passages were continued and an experiment similar to the last carried out with a vaccine lymph obtained after thirty-one passages. Titration in monkeys gave the following result:

Dilution.	Monkey. 201. 31st calf passage.	202. Alternate calf-monkey passage.
1/100,000	+	+
1/150,000	S +	+
1/200,000	O	+
1/250,000	O	S +
1/300,000	O	O

Again the lymph obtained after serial calf passage was lower in titre than that obtained after alternate calf-monkey passage.

The immunizing power of each lymph was tested.

Monkey No. 203 received 10 M.I.D. 31st passage lymph.
,, 204 ,, 10 ,, alternate passage lymph.

Fourteen days later their immunity was tested with the following result (Table II):

TABLE II.

Dilution.	Monkey. 203.	204.	Non-immune control.
Undiluted	V.S. vesicle	V.S. vesicle	—
1/10	P	P	++++
1/100	P	P	+++
1/1000	O	V.S. P	++
1/50,000	—	—	+
1/100,000	—	—	O

The reaction in the immunized monkeys appeared twenty-four hours after insertion and disappeared without leaving a trace on the third day.

Third experiment.—In this experiment, vaccine lymph obtained after thirty-three consecutive calf passages was tested. The result of titration in monkeys was as follows:

Dilutions.	Monkey. 231. 33rd calf passage.	220. Alternate calf-monkey passage.
1/140,000	+	++++
1/180,000	+	++++
1/200,000	V.S. +	++++
1/240,000	O	+++
1/300,000	O	++

A second titration was performed as the end point of No. 220 did not appear to have been reached. In the case of this titration, both were done on the same monkey.

Dilution.	33rd passage.	Alternate passage.
1/100,000	+++	—
1/120,000	++	—
1/140,000	+	—
1/180,000	O	—
1/200,000	O	+++
1/300,000	—	+
1/340,000	—	+
1/380,000	—	O

Once again the titre of the vaccine lymph obtained after serial calf passage was lower than that obtained after alternate calf-monkey passage.

The immunizing power of each vaccine was tested with the following result:

Monkey No. 238 received 50 M.I.D., 33rd, passage.
,, 237 ,, 100 ,, ,,
,, 240 ,, 50 ,, alternate passage.

The immunity conferred, tested fourteen days later, is shown in the following table :

Dilution.	Monkey.			
	237.	238.	240.	Non-immune control.
Undiluted	V.S. vesicle	V.S. vesicle	V.S. vesicle	—
1/5	do.	do.	do.	—
1/10	P	P	do.	—
1/240,000	—	—	—	+++
1/300,000	—	—	—	++

The reactions of the immunized monkeys appeared as usual within twenty-four hours of insertion and all trace had disappeared in three days.

This result confirms that of the former two experiments that there is no loss of antigenic power when the virus is propagated by consecutive calf passage, but there is considerable loss of activity.

DISCUSSION.

The deterioration of vaccine lymph on constant passage through the same vaccinifer has long been observed, but there is no account in the literature of any exact experimental work to ascertain the degree of degeneration.

Ballard (1868) quotes Kinglake as having stated in the year 1814 that the vaccine lymph then in use had deteriorated in consequence of a long succession of human passages. Estlin (1838) says " Having been engaged in vaccinating (at one time rather extensively) for thirty years, I have watched with regret a decided decline in the activity of the virus." Ceely (1840) found it " impossible to deny the occurrence of the fact." Badcock (1845) says that " Medical men repeatedly find in practice that vaccination with the old stock of lymph requires to be repeated more than once, and occasionally as often as four times, ere a genuine vesicle is produced, and that even when they have used great care in the selection of the lymph they employ." Ballard reviewed the evidence regarding the alleged degeneration of vaccine lymph and concluded that it was a fact. Seaton (1868) says that " When primary cow-lymph has been transmitted through several cows in succession the affection it produces in the animals appears, according to the accounts given, to be much less active." In more modern times we find the same opinions expressed. Blaxall (1921) states that lymph carried on from calf to calf always tends to deteriorate. Lentz and Gins (1927) say that animal vaccine requires earlier renewal than human. By the choice of the best pustules only for seed, can the virulence of the original lymph be maintained for long, but gradually a degeneration sets in. Cunningham (1927) passaged vaccine lymph through six calves in succession and found that the insertion success-rate of the resulting vaccine lymph when used on children, showed a definite and progressive though slight loss of potency with each sub-passage.

The experiments described in this paper confirm these observations and show that the loss of potency is due to a drop in titre but is not accompanied

by an appreciable loss of protective power, at any rate after thirty-three successive passages. Incidentally, the findings described in a former paper by one of us (Clearkin, 1929) that only small quantities of vaccine lymph are required to induce immunity against very large quantities, are confirmed. The third experiment showed that 50 M.I.D. of lymph protected against 300,000 M.I.D.

Vaccine lymph in the present day is undoubtedly more potent than it was during the nineteenth century, failure to "take" is not common and Gordon (1925) found that of seven samples of lymph used in England, the titre of two was 1/100,000, three 1/10,000, one 1/1,000 and one was inactive in a dilution of 1/100. Contrast this with the state of affairs during the last century when difficulty was frequently experienced in obtaining lymph of sufficient potency to ensure a successful result. It was not until early in the present century that Calmette and Guerin (1901) showed how deterioration of lymph stocks resulting from a succession of calf passages could be prevented, and a vaccine lymph of high potency obtained by interposing a rabbit passage. This method of culture was eventually adopted in vaccine lymph institutes all over the world in order to obtain lymph of high potency. It appears to have been introduced into the English Government Lymph Establishment about the year 1907. Blaxall (1921) says "this is the method I have used for many years to maintain the potency of lymph supplemented by the use of high class lymph obtained direct from the calf. The last stock obtained from Cologne was in 1907 and since then the stock has been renewed entirely by these methods."

The low potency of the vaccine lymph in use during the nineteenth century probably explains the absence of nervous complications following vaccination during that period and it is curious that not long after alternate animal passage as a means of raising the potency of lymph was introduced into England about the year 1907, the first case of post-vaccinal encephalitis was observed—in 1912. A high-grade virus will generalize more rapidly than a low-grade one and it is reasonable to suppose that the greater the invasive power of a virus the greater the likelihood of subsequent complications.

CONCLUSIONS.

1. Serial passage of vaccine lymph through a number of calves results in marked loss of activity.
2. This loss of activity is not accompanied by loss of antigenic power.
3. Small quantities of vaccine lymph immunize a monkey against very large quantities.
4. The absence of nervous complications following vaccination during the nineteenth century may be due to the fact that vaccine lymph during that period was not so potent as at the present day.

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A NOTE ON THE TOXICITY OF AMYL-META-CRESOL.

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ACCORDING to Leonard (1924), hexyl resorcinol with a phenol coefficient of 46–52 is one of the most powerful non-toxic organic germicides ever described. Coulthard, Marshall and Pyman (1930), however, described the preparation of amyl-meta-cresol, a relatively non-toxic substance, having a phenol coefficient of about 250. It is proposed in this paper to give an account of some of the experiments on which the claim for the relative non-toxicity of amyl-meta-cresol is based, and also to include some results obtained in an investigation of the toxicity of hexyl resorcinol.

EXPERIMENTAL.

The median lethal dose (M.L.D.) as defined by Trevan (1927) was determined for both compounds when administered orally and by subcutaneous and intraperitoneal injection. Mice were used except where the compounds were given orally, when rats were found to be more convenient. The results are given in Table I:

TABLE I.—*Toxicity of Amyl-meta-cresol and Hexyl Resorcinol.*

Substance.	Form.	Method of administration.	Animal.	Median lethal dose. gm./Kg.
Amyl-meta-cresol . 25 per cent.	emulsion	Oral	Rat	2·5
,, . 25 ,,	solution in olive oil	,,	,,	4–4·5
,, . 20 ,,	,, ,,	Subcutaneous	Mouse	Approx. 7·5
,, . 1 ,,	emulsion	Intraperitoneal	,,	0·15
,, . 5 ,,	solution in olive oil	,,	,,	1·25
Hexyl resorcinol . 25 ,,	emulsion	Oral	Rat	0·25
,, ,, . 25 ,,	solution in olive oil	,,	,,	1·5
,, ,, . 5 ,,	,, ,,	Subcutaneous	Mouse	0·75–1·0
,, ,, . 1 ,,	emulsion	Intraperitoneal	,,	0·05
,, ,, . 5 ,,	solution in olive oil	,,	,,	0·2

A post-mortem examination of the rats which died following the oral administration of these compounds revealed only a slight reddening of the intestines and stomach lining. Histological sections showed a slight sub-epithelial infiltration of the mucosa of the stomach and duodenum, which was so slight as to be negligible. The heart, liver, spleen and kidneys were also examined, but, with the exception of the kidneys, no definite changes were observed. The kidneys showed very slight damage to the tubules in the case of amyl-meta-cresol and a little more in the case of hexyl resorcinol.

Intravenous Injection.

Mice were found to tolerate a dose as large as 1 c.c. per 20 gm. of a saturated solution in saline, whilst up to 100 c.c. of the same solution had no depressor action on rabbits arranged for recording blood-pressure.

Inunction.

Strong solutions of both amyl-meta-cresol and hexyl resorcinol when applied to small areas of depilated skin on the backs of mice produced a wrinkling and reddening of the epidermis which was followed by local necrosis and the formation of a dry scab. After a few days, the period varying with the concentration of the solutions, the mice died without any marked change in the painted area. Toxic solutions of lower concentration produced no necrosis and no scab formed, the only change being the slight wrinkling and reddening of the skin. The average survival period for groups of four mice is given in Table II.

TABLE II.—*Toxicity by Absorption Through the Skin of Mice.*

Substance.	Strength of solution.	Average survival period.
Amyl-meta-cresol	100 per cent.	2 days
,,	40 ,,	5 ,,
,,	20 ,,	7½ ,,
,,	10 ,,	13 ,,
,,	5 ,,	Over 14 days
Hexyl resorcinol	40 ,,	2 ,,
,, ,,	20 ,,	5 ,,
,, ,,	10 ,,	7½ ,,
,, ,,	5 ,,	14 ,,

DAILY DOSES OVER LONG PERIODS.

A. *Rabbits.*

Leonard (1924) was able to feed 0·5 gm. of hexyl resorcinol daily to rabbits without toxic effect. We have been able to confirm this, having fed 0·6 gm. and 0·3 gm. respectively for 25 days without observing any toxic symptoms. The urine and fæces were examined daily for blood, albumin, pus-cells, casts, etc., with negative results. In a parallel test in which 0·6 gm. of amyl-meta-cresol was fed under exactly similar conditions no toxic symptoms were observed and the urine findings were negative.

B. *Rats.*

Rats in groups of six or over received daily doses of amyl-meta-cresol or hexyl resorcinol in olive-oil solutions. The doses used were 1·0 gm., 0·5 gm.,

0·25 gm. and 0·1 gm. per kgm. body-weight. The solutions were administered by stomach-tube under light ether anæsthesia, and the urine and fæces separately collected and examined for albumin, blood, epithelial cells, pus-cells and casts.

Of the animals receiving 1 gm./kg. amyl-meta-cresol daily, three died after 10, 10 and 12 days respectively, whilst the remaining three were still alive after 37 days, when the treatment was discontinued. All those receiving smaller doses survived the whole experimental period of 37 days. Hexyl resorcinol was slightly more toxic ; the rats receiving 1 gm. kg. all died after 3, 5, 5, 7, 8 and 8 days respectively. The rats receiving 0·5 gm./kg. survived 8, 9, 12, 17, 17 and 19 days. The smaller doses proved non-lethal even after 37 days' treatment.

The daily urine examination suggested that there was some very slight renal damage with the largest doses of both substances, there being present after the 1·0 and 0·5 gm. doses slight traces of albumin and minute traces of blood-pigment. No pus-cells, casts or blood-cells were seen in any animal and only a few squamous epithelial cells.

The fæces of the rats receiving the 1·0 gm. dose showed a trace of blood-pigment.

At intervals during the investigation animals were killed, and the liver, spleen, kidneys, heart, stomach and intestines removed for histological examination. The organs of those rats which died were also examined. This examination showed a very slight sub-epithelial infiltration of the mucosa of the stomach and duodenum in the rats receiving the larg. doses of both substances,although the damage was so slight as to be almost negligible. The kidneys of the animals which died following the daily dose of 1·0 gm. per kg. of amyl-meta-cresol showed very slight damage to the tubules, and a little more damage was observed following the same dose of hexyl resorcinol.

ABSORPTION AND ELIMINATION.

The fact that amyl-meta-cresol can be taken over long periods in very large doses without harmful effect suggested that it must be rapidly absorbed and eliminated. To get some direct evidence on this point, a number of rats were given, by stomach-tube, a dose of 250 mgm. in 25 per cent. aqueous emulsion. Then at intervals varying from 5 minutes to 96 hours, two or three of these animals were killed, the stomach and intestines immediately removed and their amyl-meta-cresol content estimated chemically.

The method of estimation, which was devised by Mr. G. F. Hall, B.Sc., is as follows :

Shred the stomach and intestines finely with scissors, wash into a steam distillation apparatus and steam distil until the fluid passing over is no longer opalescent. This requires 500–800 c.c. of distillate. Add to the distillate 1 c.c. N/1 NaOH per 100 c.c. to retain the fatty acids and extract four times with 75 c.c. of petrol ether. Wash combined extracts with 25 c.c. of water and extract this wash-water with petrol ether. Evaporate the combined petrol ether extract at 30–40° C. to about 5 c.c., at which volume complete the evaporation *in vacuo* to constant weight of amyl-meta-cresol.

It was found that rapid absorption and elimination had taken dlace, less

than 50 per cent. of the amyl-meta-cresol administered remaining at the end of 24 hours, and less than 10 per cent. at the end of 70 hours. The average values obtained by using a large number of animals are shown graphically in Fig. 1. This result is of particular importance when it is remembered that amyl-meta-cresol promises to be a useful urinary antiseptic, and it demonstrates the necessity of repeated doses at frequent intervals if it is desired to maintain a high concentration in the urine.

Separate analyses of the stomach, small and large intestines showed that the absorption was principally from the stomach.

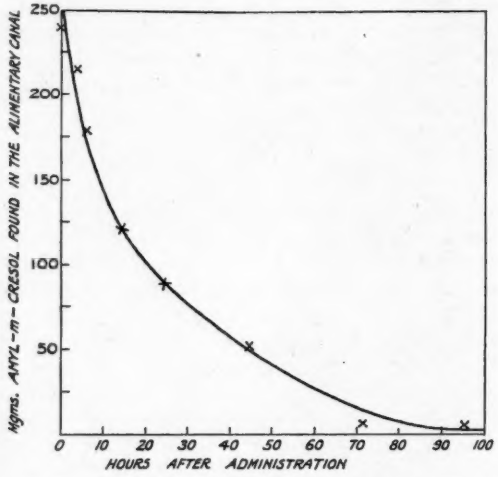

FIG. 1.—The rate of absorption of amyl-meta-cresol from rats' alimentary canal. Dose 250 mgm.

INHALATION.

In the previous paper Coulthard reports the action of the vapour of amyl-meta-cresol on various organisms. The plate cultures discussed by him were exposed to the vapour in a fume chamber, which also contained a number of rats in an open wire cage. The fume chamber had a capacity of 6 cubic feet, and the amyl-meta-cresol was vaporized in an open dish over a small bunsen flame at a standard rate of 0·5 c.c. in 60 minutes. Rats were exposed to this atmosphere for $\frac{1}{2}$, 1 and 2 hours daily for periods of 5, 10, 20 and 30 days. The animals were killed and the lungs examined microscopically. The lungs from one of the animals exposed to the largest dose for 30 days showed a thickening of the alveolar walls, and in one other animal from the same group there was evidence of a very slight inflammation of the bronchial tubes. The lungs from the rest of the animals showed no abnormality.

SUMMARY.

1. The toxicity of amyl-meta-cresol and hexyl resorcinol has been determined on rats and mice. Both compounds are relatively non-toxic, the cresol being less toxic than the resorcinol compound.

2. Neither compound has any marked cumulative action, as it is possible to administer large doses daily without toxic symptoms.

3. Toxic absorption occurs when an oily solution of either substance is applied to the skin of mice, amyl-meta-cresol being about half as toxic as hexyl resorcinol.

4. Amyl-meta-cresol is rapidly absorbed and eliminated.

5. Amyl-meta-cresol has no deleterious action on the lungs when inhaled daily by rats for long periods.

My thanks are due to Prof. E. C. Dodds, of the Courtauld Institute of Biochemistry, for his advice and for carrying out the histological examinations; to Dr. Pyman for his advice and criticism, and to my colleagues in the Pharmacological Department, Boots Pure Drug Co. Ltd., who have assisted at various stages in the work.

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THE DISINFECTANT AND ANTISEPTIC PROPERTIES OF AMYL-META-CRESOL.

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IN their paper, "The Variation of Phenol Coefficients in Homologous Series of Phenols," Coulthard, Marshall and Pyman (1930) state, "Of the n-amyl cresols, 4-n-amyl-m-cresol has been studied in some detail, and has been found to have high phenol coefficients. Since in addition its toxicity is comparatively low, it may prove to be of value in medicine." The following paper places on record some of the experiments upon which this statement was based, and also some results obtained subsequently.

Amyl-meta-cresol is relatively insoluble in water, hence in carrying out

tests it is necessary to use dilute alcohol, dilute alkali or some other menstruum. In these tests a weighed amount was dissolved in the necessary amount of undiluted alcoholic solvent or in N/10 NaOH and the solution so obtained diluted with distilled water. It is difficult to get a solution by shaking the pure amyl-meta-cresol with dilute solvent.

A. DETERMINATIONS OF THE RIDEAL WALKER COEFFICIENT USING VARIOUS ORGANISMS.

Similar results are obtained whether the solvent used is alcohol or alkali. The exact technique adopted in preparation of the solutions was :

(*a*) Addition of a 1 per cent. solution in rectified spirit to N/10 NaOH and subsequent dilution with sterile distilled water, until at a dilution of 1/25,000 the NaOH concentration was N/250 to N/500.

(*b*) Addition of a 1 per cent. solution in rectified spirit to more alcohol and subsequent dilution with sterile distilled water, until at a dilution of 1/25,000 the concentration of alcohol was 16 to 20 per cent.

The dilution of disinfectant used in calculating the Rideal Walker coefficient is that which kills the test organism in $7\frac{1}{2}$ minutes but not in 5 minutes ; this is compared with the phenol dilution having the same effect. Tests were carried out to ensure that any percentage of solvent used would not by itself be lethal in anything like this period. It was found that 25 per cent. ethyl alcohol was not lethal under test conditions in 30 minutes and N/100 NaOH (several times stronger than test dilutions) not in 20 minutes.

Coefficients obtained in a series of tests were :

(i)	Test organism	*B. typhosus*	250 to 300
(ii)	,,	*B. coli*	203
(iii)	,,	*Staphylococcus aureus*	222
(iv)	,,	*Streptococcus fæcalis*	200

In all four cases a 1/10,000 dilution of the disinfectant in N/200 NaOH was diluted with sterile distilled water as required.

B. DETERMINATIONS OF THE INHIBITIVE EFFECT.

These have been carried out using the following technique : Calculated amounts of solutions of amyl-meta-cresol in alcohol or alkali were added to 10 c.c. tubes of sterile trypsin broth. A loopful of a 24-hour-broth culture of the organism concerned was then added and the presence or absence of growth noted after 48 hours.

The smallest amounts of amyl-meta-cresol preventing growth were :

For *B. coli*	1/20,000
,, *B. typhosus*	1/30,000
,, *B. pyocyaneus*	1/10,000
,, *Staphylococcus aureus* . . .	1/50,000 to 1/75,000
,, streptococci (fæcal) . . .	1/40,000
,, *B. smegmatis*	1/100,000

C. DETERMINATION OF THE EFFECT OF THE ADDITION OF VARIOUS SUBSTANCES UPON THE EFFICIENCY OF AMYL-META-CRESOL.

(1) *Addition of Serum.*

(*a*) *Lethal efficiency.*—A solution of 1/10,000 amyl-meta-cresol in 20 per cent. isopropyl alcohol under test conditions killed a strain of fæcal streptococci in under 1 minute; the addition of 2·5 per cent. of ox-serum increased the killing-time to 3 minutes.

(*b*) *Inhibitive efficiency.*—A dilution of 1/75,000 amyl-meta-cresol in trypsin broth prevented the growth of a strain of *Staphylococcus aureus*, 1/20,000 of a strain of *B. coli*. Under otherwise identical conditions the addition of 10 per cent. ox-serum rendered the addition of 1/20,000 and 1/15,000 respectively necessary to prevent growth.

(2) *Addition of Acid or Alkali.*

The addition of more than a little alkali to solutions of amyl-meta-cresol appears to reduce lethal efficiency considerably. Thus when two solutions of 1/20,000 were prepared, one in N/400 NaOH and one in N/200, the former solution killed the test organism (*B. typhosus*) much more rapidly.

(3) *Addition of Emulsifying Agents.*

In our experiments the preparation of amyl-meta-cresol emulsions has considerably lowered the phenol coefficient. This is in accordance with the finding by B. Hampil (1928) and others that the addition of soaps to some similar cresols and phenols markedly lowers their efficiency.

D. AMYL-META-CRESOL VAPOUR: DETERMINATION OF THE EFFICIENCY OF THIS AS AN INHIBITANT OF GROWTH.

Technique of tests.—The organisms under test were heavily sown from young cultures, usually 24-hour cultures in broth, on to the surface of nutrient agar in 3-inch Petri dishes. Small sterile filter-papers were then inserted into the lids of the dishes and a standardized three drops of antiseptic dropped on each filter-paper. The bottom halves of the dishes were then inserted in the lid in the usual manner, and after incubation at 37° C., agar above, filter-papers below, the presence or absence of growth was read off. Controls were put on without antiseptic and with acetone only.

Dilutions were prepared in acetone. Amyl-meta-cresol gives similar results with acetone, rectified spirit, or isopropyl alcohol. Thirty-eight volatile antiseptics were compared by this test. The five most potent substances tested were amyl-meta-cresol, formaldehyde, oils of cinnamon and cassia and thymol, the actual order of preference varying with the organisms tested.

Amyl-meta-cresol was found to be markedly superior when tested against three cultures of acid-fast bacteria. The three cultures used were: (*a*) *B. smegmatis*, (*b*) *B. phlei*, (*c*) *B. "Mist" Moeller*. Table I gives the results of these experiments. It will be noted that although growth occurred with

25 per cent. eucalyptus oil and guaiacol, 10 per cent. creosote and phenol, and 1·25 per cent. oil of cassia, thymol and liq. formaldehydi, 1 per cent. amyl-meta-cresol prevented it.

TABLE I.—*Inhibition of Growth of Acid-Fast Bacteria by Vapour.*

Phenol, oil, etc.	3 drops (25%).	3 drops (10%).	3 drops (2·5%).	3 drops (1/80).	3 drops (1%).
None (control)	+	+	..	+	+
Eucalyptus oil	+	+	+	..	..
Guaiacol	+	+	+	..	..
Phenol	—	+	+	..	..
Creosote	—	+	+	..	..
Formaldehyde (B.P. sol. 36–38 per cent.)	—	—	—?	+	+
Oil of cassia	—	—	—?	+	+
Thymol	..	..	—	+	..
Amyl-meta-cresol	—	—	—	—	—

? denotes that there was possibly slight growth.
+ indicates growth of one or more of the three organisms.
— indicates complete absence of growth.

1 in 120 amyl-meta-cresol in acetone usually shows growth.

Confirmatory evidence as to the great value of the vapour as an antiseptic was obtained in a series of tests in which organisms were exposed to the vapours obtained by heating amyl-meta-cresol and, as a control, creosote, over a small bunsen flame in a fume cupboard. The capacity of the cupboard was about 6 cubic feet, definite amounts of disinfectant were heated, and so far as possible all was vaporized during the period of the test.

The following organisms were sown upon nutrient agar in Petri dishes, exposed to the vapour for the specified time and then incubated at 37° C.: *B. pyocyaneus*, *B. coli*, *B. typhosus*, *B. smegmatis*, *B. phlei*, *Staphylococcus aureus* (2 strains), *Streptococcus fæcalis* (3 strains).

With 0·5 gm. creosote vaporized over a period of 1 hour all organisms grew. Using 0·25 gm. amyl-meta-cresol vaporized over a period of 30 minutes the acid-fast bacteria did not grow and the cocci were either prevented from growing or grew very weakly.

Using 0·5 gm. amyl-meta-cresol vaporized over a period of 1 hour only *B. coli* and *B. pyocyaneus* grew.

Agar plates sown with *Aspergillus niger* and blood-agar plates sown with pneumococci types 1, 2 and 3, three strains of *Streptococcus hæmolyticus* or *Neisseria catarrhalis* failed to develop after 1 hour's exposure to amyl-meta-cresol vapour, although creosote treatment usually failed to affect growth.

These fume cupboard tests were carried out in conjunction with Mr. W. A. Broom, B.Sc., A.I.C., and toxicity tests were carried out by him simultaneously (*cf.* p. 327).

E. AMYL-META-CRESOL AS A URINARY DISINFECTANT.

(1) *Freedom from Toxicity.*

Gelatine capsules were prepared each containing 0·15 gm. of amyl-meta-cresol in olive oil. A gradually increasing number of these capsules were taken per day until ten was reached without ill-effect. The ten capsules were taken in pairs at 9.30 a.m., 11.50 a.m., 2.30 and 11.30 p.m.

(2) *Evidence of Disinfecting Effect.*

Samples of urine passed on the day the ten capsules were taken were collected in sterile receptacles and tested for disinfecting efficiency. The samples were—

U2 collected 7.30 a.m. (no capsule taken).
U3 ,, 5.30 p.m. (six capsules taken).
U4 ,, 4.30 a.m. next day (ten capsules taken).

These were tested both at the pH at which they were passed and neutralized to litmus, against a series of other samples from three sources: 10 c.c. amounts of each urine were sown with equal volumes of a broth culture of *B. typhosus* and plated after 24 hours' incubation at 37° C.

These results are given in Table II:

TABLE II.—*Effect of Amyl-meta-cresol as a Urinary Disinfectant.*

Tube.	Contents.	Capsules taken.	pH.	Colonies, 24 hours.
1	Control urine, R. G. P—	0	5	Uncountable
2	,, ,, P. H. D—	0	5 to 6	,,
3	,, ,, C. E. C—	0	5	,,
4	,, ,, R. G. P—	0	Neutral to litmus	,,
5	,, ,, P. H. D—	0	,, ,,	,,
6	,, ,, C. E. C—	0	,, ,,	,,
7	,, ,, U2	0	5 to 6	,,
8	A.-M.-C. urine, U3	6	5 to 6	0
9	,, ,, U4	10	5 to 6	0
10	Control urine, U2	0	Neutral to litmus	Uncountable
11	A.-M.-C. urine U3	6	,, ,,	0
12	,, ,, U4	10	,, ,,	0

F. AMYL-META-CRESOL AS A PREVENTIVE OF MOULD.

Amyl-meta-cresol in varying amounts was added to tubes of trypsin broth and the tubes sown with various moulds, including species of *Sterigmatocystis*, *Aspergillus*, *Penicillium* and one *Thamnidium*. All the moulds tested were inhibited by a dilution of 1/30,000.

Using the vapour efficiency test as detailed in Section D, three drops of a 2·5 per cent. solution in acetone showed restraining power on mould growth,

three drops of a 5 per cent. solution prevented the growth of all species of mould tested.

SUMMARY.

1. These results appear to justify the claim that amyl-meta-cresol is a disinfectant showing high lethal and inhibitive efficiency against all the bacteria used in our tests, also against moulds.

2. Rideal Walker (phenol) Coefficients of 200 and over have been obtained using *B. typhosus*, *B. coli*, *Staphylococcus aureus* and *Streptococcus fæcalis*.

3. In broth amyl-meta-cresol dilutions up to or beyond 1/70,000 show inhibitive effect, and dilutions of 1/20,000 to 1/30,000 prevent the development of many bacteria and moulds.

4. Amyl-meta-cresol vapour, either vaporized by heat or allowed to vaporize spontaneously, is effective in extreme dilution in preventing the growth of and ultimately killing many organisms. Amongst bacteria so affected are *B. smegmatis* and other acid-fast organisms, pneumococci types 1, 2 and 3, *Streptococcus hæmolyticus* and moulds.

5. Amyl-meta-cresol appears to confer bactericidal properties on urine.

My thanks are due to Dr. F. L. Pyman for his criticisms and suggestions; to Dr. J. Marshall for assistance on the chemical side; to Miss D. Wainman for much careful work at the bench; and to Miss O. E. Antill for her help in getting the paper into shape.

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FURTHER OBSERVATIONS ON QUALITATIVE DIFFERENCES IN ANTITOXIC FRACTIONS PREPARED FROM THE SAME SAMPLE OF PLASMA.

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In an earlier communication (1931) we have shown that a series of globulin fractions of different antitoxic quality may be produced from a single sample of plasma by means of salting-out methods. The plasma used in our experiment possessed a high *in vivo/in vitro* ratio, and according to animal titrations contained 950 units of diphtheria antitoxin as compared with 625 units per c.c. obtained by the flocculation test. By successive precipitations with solid ammonium sulphate, eight globulin fractions were prepared from this material, and were tested for antitoxic content by the intracutaneous method in guinea-pigs and by flocculation with toxin *in vitro*. By means of these tests we have compared the quality of the antitoxin associated with different portions of the serum globulins, using the *in vivo/in vitro* ratio as the basis of comparison. The value of this ratio was maximal in the first fraction, *i. e.* that separated by the smallest quantity of ammonium sulphate, and a steady decrease occurred throughout the series from 2·69 in this fraction to 0·93 in the last.

In order to determine whether this phenomenon is a characteristic of all diphtheria antitoxic sera, other samples have now been fractionated and examined in a similar manner. The method adopted for fractionation was identical with that used previously by us, and as before, the protein content of each fraction was determined by means of micro-Kjeldahl nitrogen estimations.

In these experiments samples of plasma were selected for which the value of the ratio was greater than, equal to and less than, unity. The first of these samples (A) contained 800 units according to animal titration and 600 units as measured by the flocculation test, giving a serum ratio of 1·33. The antitoxic values of the fractions as determined by both methods of testing, recorded in Table I, are correct to within $\pm$ 5 per cent. It will be seen that in the first three fractions the value of the ratio is higher than that of the original plasma, and a progressive decrease occurs throughout the series to the sixth fraction, with a slight increase from this to the eighth fraction. Values for the purity of the antitoxin are shown in Fig. 1, and are expressed as antitoxic units per gramme of protein : in curve 1 the antitoxic values were obtained from animal tests and in curve 2 from flocculation tests. Since the value of the ratio in

no place decreases to unity, the entire course of curve 1 lies above that of curve 2.

The second sample of plasma (B) was characterized by an *in vivo/in vitro* ratio of unity, and a value of 625 units per c.c. was obtained by both methods of testing. The antitoxic values of successive ammonium sulphate fractions

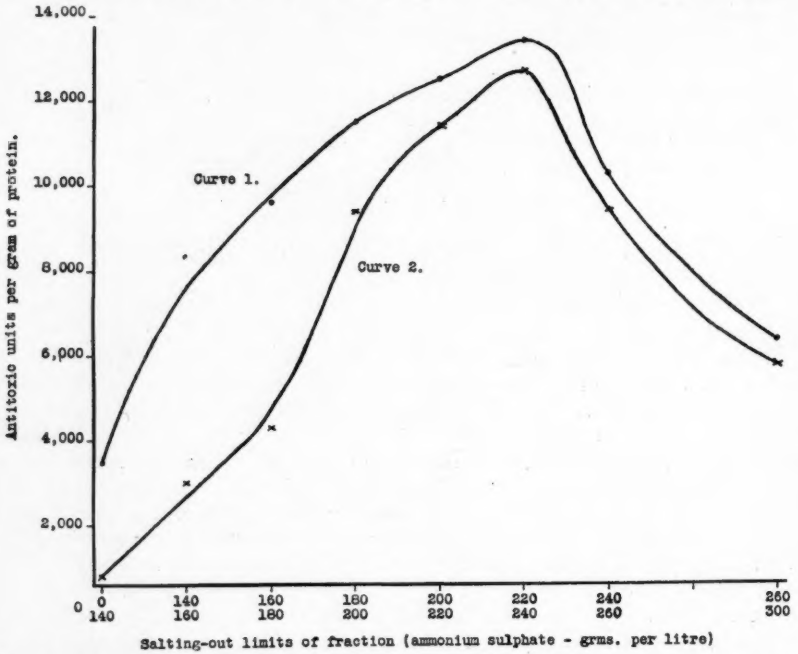

FIG. 1.—Showing the purity of diphtheria antitoxin in a series of globulin fractions prepared from plasma A by salting-out with ammonium sulphate. Curve 1: Antitoxic values from *in vivo* titrations. Curve 2: Antitoxic values from *in vitro* titrations.

TABLE I.—*Showing the* in vivo *and* in vitro *Values of a Series of Globulin Fractions Prepared from a Sample of Plasma* A, *for which the* in vivo *Value was* 800 *units and the* in vitro *Value* 600 *units. Serum Ratio* 1·33.

No. of fraction.	Precipitation limits ammonium sulphate grammes per litre.	Antitoxic value. *In vivo.*	Antitoxic value. *In vitro.*	Serum ratio.
1	0 to 140	225	54	4·24
2	140 ,, 160	160	58	2·76
3	160 ,, 180	425	190	2·23
4	180 ,, 200	525	430	1·22
5	200 ,, 220	480	440	1·10
6	220 ,, 240	275	260	1·06
7	240 ,, 260	125	115	1·09
8	260 ,, 300	53	48	1·10

are given in Table II and purity curves in Fig. 2. The value of the serum ratio in the first three fractions again exceeds that of the original plasma, and decreases progressively from 1·67 in the first fraction to 0·94 in the seventh.

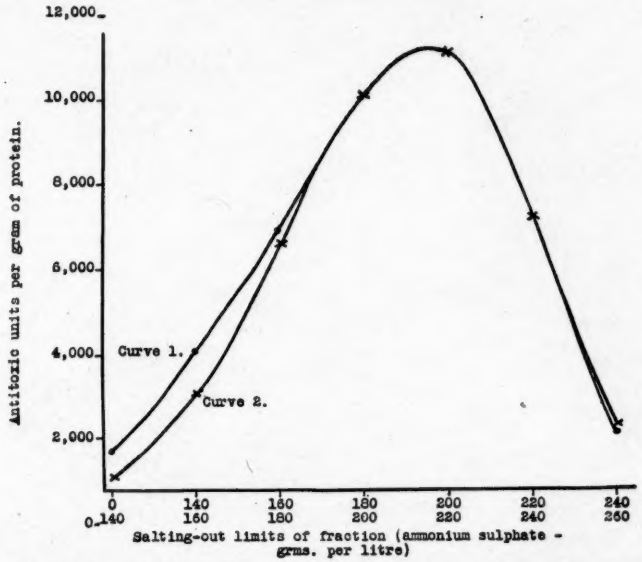

FIG. 2.—Showing the purity of diphtheria antitoxin in a series of globulin fractions prepared from plasma B by salting-out with ammonium sulphate. Curve 1: Antitoxic values from *in vivo* titrations. Curve 2: Antitoxic values from *in vitro* titrations.

TABLE II.—*Showing the* in vivo *and* in vitro *Values of a Series of Globulin Fractions Prepared from a Sample of Plasma* B, *for which both the* in vivo *and* in vitro *Values were* 625 *units. Serum Ratio* 1·00.

No. of fraction.	Precipitation limits ammonium sulphate grammes per litre.	Antitoxic value		Serum ratio.
		In vivo.	*In vitro.*	
1	0 „ 140	45	27	1·67
2	140 „ 160	55	42	1·31
3	160 „ 180	50	48	1·04
4	180 „ 200	170	170	1·00
5	200 „ 220	90	90	1·00
6	220 „ 240	26	27	0·96
7	240 „ 260	16	15	0·94

Another series of fractions was prepared from a plasma (C), for which the *in vivo* value was 525 units and the *in vitro* value 825 units, giving a serum ratio of 0·65. The fractions of larger particle size were again characterized by showing a higher ratio than that of the parent material.

The figures in Table III show, however, that the serum ratio decreases to a minimum value in the fourth solution, with a definite increase from this to the fifth and sixth fractions. A precipitate was prepared by addition of 140 gm. of ammonium sulphate as usual, but it was found to contain so little antitoxin and was of such extreme opacity as to render impossible any results from *in vitro* tests.

TABLE III.—*Showing the* in vivo *and* in vitro *Values of a Series of Globulin Fractions Prepared from a Sample of Plasma* C, *for which the* in vivo *Value was* 525 *units and the* in vitro *Value* 825 *units. Serum Ratio* 0·65.

No. of fraction.	Precipitation limits ammonium sulphate grammes per litre.	Antitoxic value. *In vivo.*	Antitoxic value. *In vitro.*	Serum ratio.
1	140 to 160	35	34	1·03
2	160 ,, 180	175	235	0·74
3	180 ,, 200	220	320	0·68
4	200 ,, 220	150	280	0·54
5	220 ,, 240	62	95	0·65
6	240 ,, 260	36	42·5	0·85

These results definitely confirm our earlier observation that the *in vivo/in vitro* ratio is high for antitoxin associated with the protein of largest molecular size or aggregation. Further confirmation of this phenomenon is given in Table IV, which shows the average value of the serum ratio in a number of routine blends before and after concentration with ammonium sulphate. The tests on the individual blends were accurate to within ± 10 per cent. Each

TABLE IV.—*Showing the Average Value of the Serum Ratio for a Number of Routine Blends before and after Concentration with Ammonium Sulphate, and for Solutions of the First Precipitates in the Concentration Process.*

Description of material.	Number of batches.	Average value of serum ratio.
1. Bulked plasma before concentration	44	0·948
2. Concentrated serum obtained from 1	44	0·935
3. Solution of first ammonium sulphate precipitate from 1	18	1·08
4. Concentrated serum obtained from 3	18	0·999

batch consisted of from 50 to 100 litres of mixed plasma, and the average value for the serum ratio of forty-four such batches amounted to 0·948. After concentration this figure suffered a slight decrease to 0·935. In the process of concentration more antitoxin is removed in the first precipitation than is rejected in the waste filtrate containing the albumen, so that this decrease is due to the removal of higher-ratio material at the start of precipitation. The first precipitates from two or three batches are dissolved up together in water and constitute "recovery batches" from which some more antitoxin may be

obtained. The figure obtained as the average for eighteen such solutions was 1·08—a higher figure than that obtained for the plasma blends: after concentration the value fell to 0·999.

The reason for the slightly low values of the ratio is clear on consideration of our routine methods of testing. Daily checks are made upon the strength of the test toxins in relation to the standard unit of antitoxin. Any decrease in strength of toxin used for flocculation tests can be detected in a few hours and the necessary adjustment in test dose made immediately. Intracutaneous tests, however, cannot be made so rapidly, and several days may elapse before any deterioration in the test toxin is definitely established. It is our custom, therefore, to maintain the test dose of toxins used for *in vivo* determinations slightly above the correct standard. This is of advantage in view of the fact that it is essential that antitoxin intended for therapeutic use should be above rather than below the stated value, and all such values must be based upon the results of *in vivo* and not *in vitro* tests. It therefore follows that tests made on routine samples of serum are recorded by us at a value which may be as much as 10 per cent. below the correct value; on the other hand, tests made in scientific experiments are referred to the results of similar tests against the standard serum, and expressed in terms of the true unit.

A possible explanation of the phenomenon of high-ratio antitoxin has already been suggested by Barr and Glenny (1931). It may be that there is more than one type of antitoxin, and that in high ratio sera only a fraction of the total antitoxin flocculates with toxin *in vitro*. If this is so, then the true quantitative measure of antitoxin is obtained by animal tests. In an attempt to obtain some evidence as to which method of testing gives the true quantitative measure, we have determined the effect of fractionating a blend of high ratio diphtheria antitoxin with tetanus and *B. welchii* antitoxic plasmas. It was shown by Barr, Glenny and Pope (1931) that on blending equal volumes of three antitoxic sera some redistribution of each antitoxin occurs, and the proportions precipitated by a given quantity of ammonium sulphate, though not always identical, tend to become averaged. It appeared probable, therefore, that the distribution of the tetanus and *B. welchii* antitoxins would be averaged on blending, and that the precipitation of the diphtheria antitoxin as determined either by *in vivo* or *in vitro* tests might follow a similar course. The sample of plasma chosen was one previously fractionated by us, and contained 950 units according to animal titrations as compared with 625 units obtained by flocculation tests. Two volumes of this material were mixed with one volume of 300-unit tetanus and one volume of 350-unit *B. welchii* antitoxic plasma. The blend was diluted with an equal volume of water and fractionated with solid ammonium sulphate by our usual method.

The diphtheria antitoxic values of the fractions were determined by intracutaneous titrations and flocculation tests, and Table V gives values of the *in vivo*/*in vitro* ratio obtained for these and similar fractions prepared from the unblended plasma.

These figures show that there is a distinct tendency for the ratio of the first five fractions to be increased and that of the last three to be decreased on blending with these particular samples of plasma. This indicates that some redistribution of antitoxic protein occurred on mixing, and confirmation

TABLE V.—*Showing a Comparison of the Serum Ratio of Similar Fractions Prepared from a Sample of Plasma* D, *Alone and After Blending with Two Half-volumes of Tetanus and* B. welchii *Antitoxic Plasmas. The* in vivo *Value of the Plasma* D *was* 950 *units and the* in vitro *value* 625 *units. Serum Ratio* 1·52.

No. of fraction.	Precipitation limits ammonium sulphate grammes per litre.	Serum ratio fractions from— Single plasma.	Blend.
1	0 to 140	2·69	2·78
2	140 ,, 160	2·55	2·54
3	160 ,, 180	1·50	2·05
4	180 ,, 200	1·22	1·47
5	200 ,, 220	1·08	1·09
6	220 ,, 240	1·00	0·93
7	240 ,, 260	1·00	0·90
8	260 ,, 300	0·93	0·85

of this is given in curves 1 and 2 of Fig. 4, which show the distribution of diphtheria antitoxin in the blended and unblended dilutions. The values used are those obtained from animal tests, but similar differences occur if flocculation values are used. The curves show that the precipitation of antitoxin

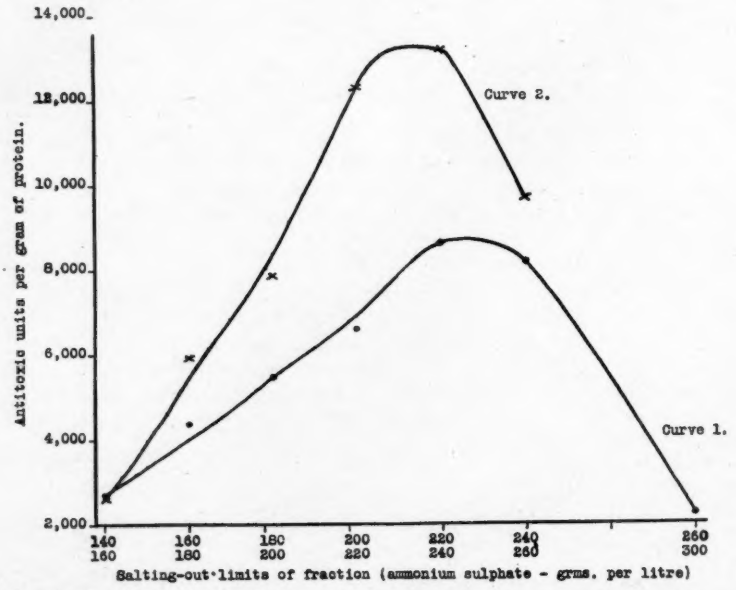

FIG. 3.—Showing the purity of diphtheria antitoxin in a series of globulin fractions prepared from plasma C by salting-out with ammonium sulphate. Curve 1: Antitoxic values from *in vivo* titrations. Curve 2: Antitoxic values from *in vitro* titrations.

from the single plasma follows a more rapid course than that from the blend: there is also delayed precipitation from the latter in the central part of the range, which does not occur in the unblended material.

The curves in Fig. 5 show the relative purity of the different antitoxins in the fractions in terms of units per gramme of protein. The purity of the diphtheria antitoxin is represented in curves 1 and 2, the values in curve 1 being those obtained from *in vivo* tests and in curve 2 from *in vitro* tests. Curves 3 and 4 show the purity of *B. welchii* and tetanus antitoxins respectively, and these values have been multiplied by 2 for convenience in scale measurements.

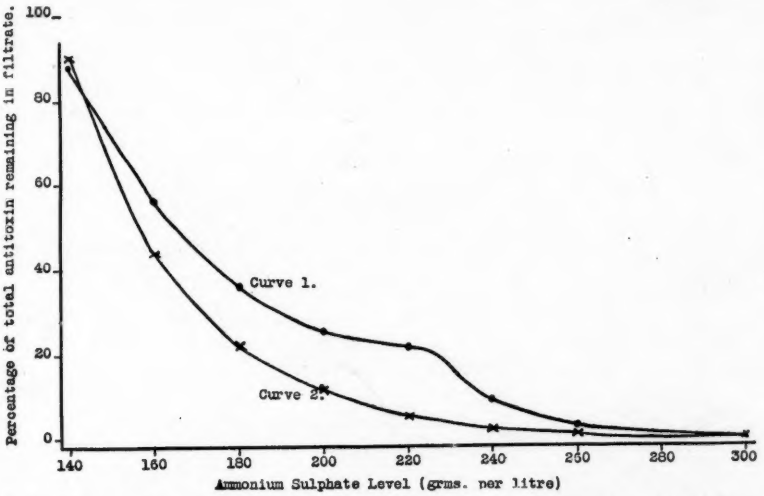

FIG. 4.—Showing the course of precipitation of diphtheria antitoxin by ammonium sulphate. Curve 1: From the blend of tetanus and *B. welchii* antitoxin plasmas. Curve 2: From the unblended material.

The tests for *B. welchii* antitoxin were performed by the intracutaneous method in guinea-pigs, and for tetanus antitoxin by intramuscular titration in mice. The general form of curves 3 and 4 resembles that of curve 2 rather than curve 1. This suggests that it is the flocculation tests which give the correct quantitative antitoxic values, since curve 1, representing animal values, follows an entirely different course, giving as its point of maximum purity the third fraction, instead of the later fractions shown in the other curves. This appears even more probable when we consider that curves 3 and 4 were obtained from the results of animal tests and might reasonably be expected to resemble curve 1 rather than curve 2, which was obtained from results of flocculation tests.

In all these experiments we have used 140 gm. per litre as our initial addition of ammonium sulphate. The relative amounts of antitoxin precipitated by this quantity of salt vary with the sample of plasma used, and it appears

probable that by making a smaller addition, a first fraction could be prepared from some samples which would possess a much higher serum ratio than any we have obtained so far. In general, and for the purpose of comparing different sera, this quantity constitutes a suitable level of concentration.

An alternative explanation of the phenomenon of high ratio was put forward by Glenny (1931), who suggested that the greater affinity of toxin over toxoid for antitoxin is particularly marked when neutralizing with sera of this kind.

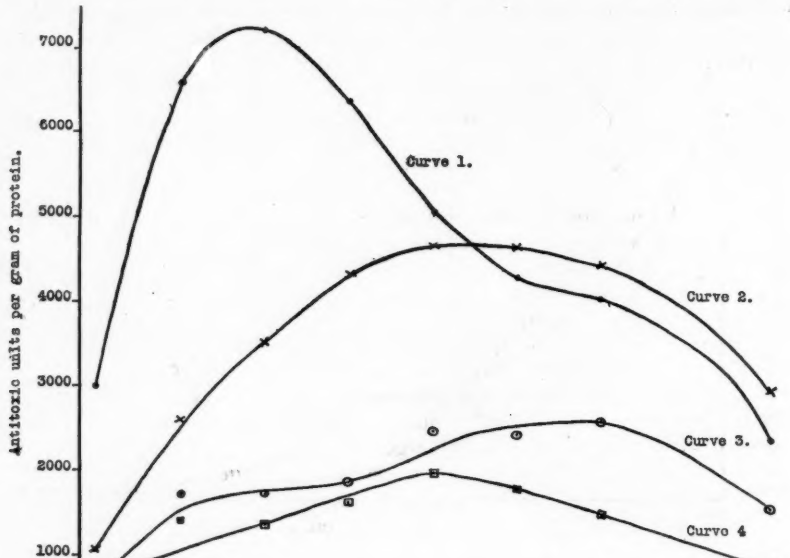

FIG. 5.—Showing the purity of antitoxins present in a series of globulin fractions prepared from a blend of plasma. Curve 1: Diphtheria antitoxin (*in vivo* values). Curve 2: Diphtheria antitoxin (*in vitro* values). Curve 3: *B. welchii* antitoxin. Curve 4: Tetanus antitoxin.

If this is so, the correct quantitative measure of antitoxic value is obtained by the flocculation test: animal tests, on the other hand, measure to a certain extent both quantity and quality of antitoxin. If high ratio antitoxin combines much more readily with toxin than with toxoid, animal tests determine how much antitoxin is necessary to neutralize all the specific toxin present in the test dose, while the non-lethal toxoid may remain unneutralized. Thus, of two sera of the same value as determined by flocculation, more of the less avid antitoxin will be needed to neutralize the test dose to a given end-point because relatively more toxoid will have entered into combination. When using sera

of very poor avidity, owing to the small affinity of the antitoxin for both toxin and toxoid, an excess of antitoxin must be present to prevent dissociation and liberation of free toxin.

It appears unnecessary to visualize two distinct qualitative measures of antitoxin. Avidity in the sense of firmness of combination with toxin may be the only quality affecting ratio. An ideal antitoxin which combines firmly with toxin may have a definitely high limiting ratio, the value of which depends on the relative quantities of specific toxin and toxoid present in the test toxin used for determining the *in vivo* value.

The majority of antitoxins possess a serum ratio of unity, and it is generally recognized that such antitoxins do not form firm and undissociable compounds with toxin. They may merely represent less avid specimens of ideal antitoxin, for it is possible to visualize grades ranging from the ideal antitoxin which would of necessity possess a high ratio and combine firmly with toxin, to antitoxin of extremely low avidity. The average sample falls into place at some point within the range.

It is by the method of fractionation that we hope to determine the upper limit of quality and approach the ideal preparation. We have shown by this method that fractions possessing different ratios may be separated from a single serum. From this we must conclude either that antitoxin of many grades exists in every sample of serum, or that removal of a fraction from a whole serum changes its properties. It would appear that a mixture of low- and high-ratio material blended in such proportions as to give a ratio of unity would not necessarily possess the average degree of avidity : a process which averages ratio may not simultaneously average avidity, and a small quantity of ideal antitoxin blended with a larger amount of relatively poor material might give a product of unit ratio but less than average avidity. Many of the sera normally encountered may fall into place within a small area of the whole possible avidity range, this area being located on either side of the average value.

Experiments now in progress show that the avidity of all fractions with a high ratio is definitely good ; the avidity of low ratio fractions is poor. These tests for avidity include :

(*a*) Intravenous injection of toxin-antitoxin mixtures into rabbits ; and

(*b*) Intracutaneous injection of mixtures at different dilutions into guinea-pigs.

We are also hoping to throw more light on the phenomenon of high ratio, by conducting intracutaneous tests against several toxins in which the relative proportions of specific toxin and toxoid are different.

SUMMARY.

1. Determination of the *in vivo*/*in vitro* ratio for a series of globulin fractions prepared from three samples of diphtheria antitoxic plasma (ratios 1·33, 1·00 and 0·65) has shown that the antitoxin in fractions precipitated by small quantities of ammonium sulphate is characterized by a ratio exceeding that of the original plasma.

2. This appears to be a general characteristic, because analysis of figures obtained from routine concentrations involving several thousand litres of plasma has shown that the average serum ratio of low ammonium sulphate fractions was 13 per cent. higher than the average for the original material.

3. Fractionation of a blend of high-ratio diphtheria antitoxin with tetanus and *B. welchii* antitoxic plasmas has shown that the purity curves for the latter antitoxins resemble that of the diphtheria antitoxin as obtained by *in vitro* and not by *in vivo* tests.

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THE MECHANISM OF THE VOGES-PROSKAUER REACTION AND THE DIACETYL REACTION FOR PROTEINS.

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Harden (1906) showed that the substance, produced from glucose in bacterial cultures, which gave the Voges-Proskauer reaction was acetylmethyl carbinol ($CH_3.CO.CHOH.CH_3$), now usually called acetoin. This substance combined with some constituent of the peptone in the cultures, when the latter were made alkaline, and the chemical interaction which took place resulted in the reddish coloration and greenish fluorescence characteristic of the reaction. He observed that the reaction began at the top of the test-tube, and concluded that oxidation was a factor in the chemical changes. He assumed that, in the presence of alkali, acetoin was oxidized by atmospheric oxygen to diacetyl ($CH_3.CO.CO.CH_3$), and found support for his assumption in the observation that peptone gave with diacetyl in the presence of alkali a reaction exactly similar to that given with acetoin, except for the fact that it was much more rapid and intense.

Later, Harden and Norris (1911), working with diacetyl, found that the amino-acid fraction of the peptone responsible for the colour change was that which contained the guanidine nucleus. They found that the red coloration, without fluorescence, was given by agmatine, arginine, creatine, dicyandiamide and guanidine-acetic acid, and concluded that it was associated with the

molecular arrangement $HN:C.NH_2NHR$, the nature of R being left undetermined. The greenish fluorescence was ascribed by them to a combination of diacetyl with unhydrolysed protein. The current view of the mechanism of the reactions under consideration may therefore be summed up as follows:

$$CH_3.CO.CHOH.CH_3 + O \rightarrow CH_3.CO.CO.CH_3$$
$$CH_3.CO.CO.CH_3 + HN:C.NH_2NHR \text{ — red colour}$$
$$CH_3CO.CO.CH_3 + \text{protein} = \text{green fluorescence.}$$

There are, however, difficulties in the way of accepting this view. In the first place if acetoin ($CH_3.CO.CHOH.CH_3$) were so very readily oxidizable to diacetyl ($CH_3.CO.CO.CH_3$), then it is to be expected that 2·3 butylene glycol ($CH_3.CHOH.CHOH.CH_3$) would, by virtue of its constitution, be oxidized to acetoin and from thence to diacetyl with almost equal readiness. In consequence it should give a reaction similar to the Voges-Proskauer reaction, whereas it does not. Although it may be converted into acetoin and diacetyl fairly readily by the aid of oxidizing agents just as acetoin may be converted by them into diacetyl, 2·3 butylene glycol is very stable even in the presence of strong alkali with free exposure to air. Again, if the Voges-Proskauer reaction took place in the manner outlined above, the many attempts which have been made to hasten it with the aid of oxidizing agents should have met with success, instead of which they have all been partial or total failures. While it is true that hydrogen peroxide, mentioned by Levine (1917), or sodium peroxide, suggested by Bedford (1929), may sometimes hasten the colour production, there is no doubt that they frequently fail to achieve this result, and sometimes prevent the appearance of any colour even when acetoin is known to be present in sufficient quantity to give the reaction strongly by the ordinary technique. With these anomalies in mind it was decided to re-investigate the mechanism of the two reactions.

THE REACTION WITH DIACETYL.

It was found at the outset that the reaction with diacetyl does not begin immediately on the addition of alkali, either with protein solutions or solutions of such bodies as dicyandiamide or arginine. Unless the amount of diacetyl present is considerable, time has to elapse before the colour begins to appear, and furthermore when it appears it comes first at the top of the fluid in the test-tube just as in the Voges-Proskauer reaction. This observation suggested that oxidation was just as essential for the diacetyl reaction as for the reaction with acetoin. The possibility of an acceleration of the reaction at the top of the tube by mere surface action had, however, to be excluded. A strong solution of protein in one case, dicyandiamide in a second and creatine in a third was mixed with an equal volume of 40 per cent. caustic soda in a boiling tube, and heated while a current of hydrogen was passed through to expel air. It was cooled, the current of hydrogen still passing, and about 1 c.c. of a 2 per cent. solution of diacetyl which had been boiled and cooled was gradually introduced into the tube. No coloration resulted in either case, even though the reagents were left in contact with one another for half an hour, an active

stream of hydrogen being maintained. On stopping the hydrogen and shaking up the contents of the tubes with air an intense red colour developed without delay.

The above findings were confirmed in a number of ways. To a strong solution of dicyandiamide an equal volume of 40 per cent. caustic soda was added in a test-tube, and the whole boiled vigorously, without shaking, to expel air. A few drops of a 2 per cent. solution of diacetyl which had been well boiled were then introduced, the vigorous boiling of the mixture being continued. No colour was visible so long as the boiling was continued and the tube kept unshaken, but on shaking up with air the red colour was immediately produced. The reaction was also carried out in boiling absolute alcohol and in cold absolute alcohol, from which air had been previously expelled, with confirmatory results.

It may, therefore, be concluded, firstly that oxygen is essential for the development of the red colour in the diacetyl reaction, and secondly, that the reaction proceeds in the presence of alkali by condensation between diacetyl or a polymeride of diacetyl and the guanidine derivative, to form a colourless body which is readily oxidized by atmospheric oxygen to a red colouring matter. The second contention follows from the fact that diacetyl survives for only a short time in a strongly alkaline solution, being converted into *p*.xyloquinone and, in consequence, the colour obtained in the above experiments on admitting air to the tubes could not be due to residual diacetyl. The colourless intermediate product in the reaction has not been isolated in a pure state, but it has been shown that it remains colourless on neutralization, and is not susceptible to oxidation by atmospheric oxygen except in alkaline solutions. The mechanism may be summarized for the sake of clearness as follows:

$CH_3.CO.CO.CH_3$ + guanidine derivative → colourless substance
or $(CH_3.CO.CO.CH_3)_n$ + ,, ,, → ,, ,,
Colourless substance + O → red substance.

The more detailed study of the mechanism of the reaction has not been attempted, and presents considerable difficulties in view of the fact that the chemicals involved are somewhat of enigmas both in regard to their reactions and their constitution. It seems probable, however, that enolization of the diacetyl is a necessary preliminary in the reaction, since one would otherwise expect benzil ($C_6H_5.CO.CO.C_6H_5$) to give a similar reaction, and, as Harden and Norris (1911) have shown, it does not. By enolization diacetyl would give rise to unsaturated and highly reactive bodies capable of condensing either among themselves or with other substances in the solution. The possibility that it is a condensation product of diacetyl and not diacetyl itself which is responsible for the reaction cannot be lost sight of. If it is such a condensation product the substance is not *p*.xyloquinone. Harden (1906) left diacetyl in contact with alkali, and found that the mixture failed, after a time, to give the reaction on the addition of peptone. This method of excluding *p*.xyloquinone is not, however, conclusive, as it is a substance very unstable in alkaline solution, rapidly absorbing oxygen from the air and darkening in colour. In order to confirm Harden's finding *p*.xyloquinone was isolated in a pure state by shaking up an alkaline solution of diacetyl with ether, the ether being decanted and

evaporated to dryness in a current of air. It is a yellow crystalline body with a typically quinonoid odour, and does not give a visible reaction with the guanidine derivatives. It was found, however, that on adding strong caustic soda to a dilute aqueous solution of *p*.xyloquinone a red colour was produced, in appearance not unlike that given by diacetyl with such substances as dicyandiamide. The colour was produced immediately on the addition of alkali and faded in a few minutes. Mere traces of *p*.xyloquinone were detectable by its odour and its behaviour in this way with alkalis.

NATURE OF SUBSTANCES REACTING WITH DIACETYL.

Harden and Norris (1911) tried a large number of substances, and found that those which gave the reaction belonged to the guanidine group. Their work has been extended in the present investigation. The bodies tried may be classified as guanidine salts, mono-substituted derivatives, symmetrical and asymmetrical di-substituted derivatives and tri-substituted derivatives of guanidine. The method generally adopted in testing was to dissolve a little of the substance to be tested in about 3 c.c. of water in a test-tube, and to add a few drops of 1 per cent. diacetyl solution, followed by 3 c.c. of 40 per cent. caustic soda, the reagents being well shaken from time to time to promote oxidation. The various substances tried were found, in general, to behave according to the group to which they belonged, as represented by their structural formulæ. The guanidine salts represented by the general formula $HN:C.NH_2NH_2X$ do not give a red coloration with diacetyl, but form a bright yellow condensation product, which deepens to a golden yellow on shaking and standing. The hydrochloride, nitrate and carbonate of guanidine were found to behave in this way.

The mono-substituted derivatives of the general formula $HN:C.NH_2NHR$ tested were amino-guanidine, nitro-guanidine, methyl guanidine, arginine and dicyandiamide. With the exception of amino-guanidine these substances all behaved similarly, giving a red coloration after a few minutes had elapsed. Heating greatly accelerated the reaction. With nitroguanidine the reaction was not strong and was produced best on heating. As mentioned above amino-guanidine does not give the red colour. It resembles the guanidine salts in its behaviour, giving, like them, a yellow colour. Both of these guanidine derivatives are very unstable in the strong alkali and evolve ammonia freely. Harden and Norris (1911) found methylguanidine completely negative, though two specimens of the hydrochloride from different sources were found to give the reaction in the present investigation. It may also be mentioned that melamine or cyanuric triamide, $N_3C_3(NH_2)_3$ gives a reaction similar in all respects to that given by dicyandiamide. According to the usual view of its constitution melamine does not contain the group $HN:C.NH_2NHR$, though it may possibly undergo intramolecular rearrangement in a strongly alkaline solution and thus give rise to the necessary grouping.

Only one symmetrical di-substituted derivative of guanidine, of the general formula $HN:C.NHRNHR$, was available for testing. This was diphenyl guanidine, which gave no reaction with diacetyl. The phenyl guanidines,

however, are very insoluble, and this may possibly account for its failure to react.

The asymmetrical di-substituted derivatives tried, of the general formula $HN : C.NH_2NRR_1$ were dimethylguanidine and creatine. These substances, when tested in the usual way, gave a red coloration, which started to develop almost immediately and rapidly deepened until it was very intense. The only tri-substituted derivative of guanidine of the general formula $HN : C.NHRNR_1R_2$ available was triphenyl-guanidine. It failed to give any reaction, but, like diphenyl-guanidine, was very insoluble. No tetra-substituted derivative of the type $HN : C.NRR_1NR_2R_3$ was tried.

We may conclude, therefore, that the substances which are known to give the diacetyl reaction with the exception of melamine may be expressed by the general formulæ $HN : C.NH_2NHR$ or $HN : C.NH_2NRR_1$. The colouring matters produced from such substances, however, do not all exhibit the same chemical properties. The differences will be made clear from a comparison of the colouring matters produced with dicyandiamide and creatine respectively.

On dilution with water the alkaline solution of the colouring matter produced from dicyandiamide changes to a permanganate tint, which is retained even though a large amount of water be added. On neutralization the colour is discharged, but reappears on the further addition of acid, resembling in this respect certain indicators. The colour is discharged by oxidizing agents such as hydrogen peroxide, and the colouring matter cannot be extracted by shaking up with ether or chloroform. A very striking property is the formation of a well-defined red magnesium lake. If to a solution of the colouring matter in weak alkali a few drops of magnesium sulphate be added, all the colour is carried down by the precipitated magnesium hydroxide, leaving the supernatant fluid colourless.

Reference will be made later to a further remarkable property of this colouring matter.

The coloured substance produced from creatine, on the other hand, appears to undergo dissociation on dilution and acquires a yellowish-red tint. On neutralization with acid the colour disappears, but requires the further addition of large quantities of concentrated acid before it makes its reappearance. It is discharged by hydrogen peroxide, but much more readily than the colour produced from dicyandiamide, and like the latter, cannot be extracted with ether or chloroform. It does not form a magnesium lake, the precipitated magnesium hydroxide falling colourless to the bottom of the tube and leaving the supernatant fluid with its original colour intact.

The presence or absence of the power to form magnesium lakes by their respective colouring matters enables the guanidine derivatives studied to be divided into three groups. The guanidine salts give yellow colouring matters which form a weak, but quite definite pink lake with magnesium. The colouring matters from nitroguanidine, methylguanidine, dicyandiamide and melamine all form strong red magnesium lakes. The colouring matters obtained with arginine, asymmetrical di-methylguanidine and creatine form no lakes with magnesium. It will therefore be seen that the substances behave according to their chemical composition. The first group is formed by substances

of the formula HN : C. NH_2NH_2X, the second group by the substances of the formula HN : C. NH_2NHR, and the third group (with the exception of arginine) by substances of the formula HN : C. NH_2NRR_1. The behaviour of the colouring matter derived from arginine is anomalous, and would suggest that this substance is not correctly represented as a mono-substituted derivative of guanidine, since it resembles creatine and asymmetrical di-methylguanidine in its behaviour. We may next pass to the consideration of a further property of the colouring matter produced from the second group of substances.

NATURE OF THE GREEN FLUORESCENCE.

Harden and Norris (1911) attributed the greenish fluorescence seen in the diacetyl reaction with protein to combination of diacetyl with some constituent of unhydrolysed protein. They found that diacetyl gave no fluorescence with the simple guanidine derivatives which they tried, and that furthermore protein, which gave the fluorescence in the unhydrolysed state, ceased to give it after hydrolysis. They also found that no fluorescence was produced even when the reaction was carried out with unhydrolysed protein if very strong alkali were used, and attributed this to hydrolysis of the protein by the concentrated alkali. The true explanation of the fluorescence is quite different. It is due to an interaction between the red colouring matter produced by the action of diacetyl on the guanidine fraction of the protein and some undetermined constituent of the protein molecule. This is very readily demonstrable by producing the red colouring matter first from a simple guanidine derivative and then adding it to a solution of protein. The colouring matter may be conveniently made by mixing diacetyl, dicyandiamide and strong alkali in a test-tube. The mixture is brought to the boil to destroy any residual diacetyl and is then cooled. A few drops of the intensely red alkaline solution thus formed are added to a solution of gelatine or of peptone, and the green fluorescence begins to appear almost at once and gradually becomes more intense. Here there is no question of diacetyl interacting with the protein; the reaction is between the protein and the red colouring matter. The fluorescence produced in this way corresponds in all respects to that produced in the Voges-Proskauer reaction or in the diacetyl reaction when protein is used. Its properties are as follows: On heating the fluorescence disappears, but reappears on cooling; on the addition of strong alkali it also disappears, but can be made to return by decreasing the alkalinity of the solution either by dilution with water or by the cautious addition of acid. It behaves, therefore, as if it were the result of some loose combination between the colouring matter and the responsible constituent of the protein molecule.

The colouring matters formed from the guanidine derivatives do not all give the fluorescence with protein solutions, and their fluorescent power appears to run parallel to their power of forming magnesium lakes referred to above. Thus we find that the colouring matters derived from the guanidine salts, which form feeble magnesium lakes, give little or no fluorescence, the colouring matters from dicyandiamide, melamine and methylguanidine, which form strong magnesium lakes, give a well-marked fluorescence, while the colouring matters from arginine, creatine and asymmetrical dimethylguanidine, which

fail to give any magnesium lake, also fail to give the fluorescence. It is remarkable that the colouring matter from arginine fails to give the fluorescence when added to a protein solution, and in this fact is to be found the explanation of the failure of hydrolysed protein to fluoresce when the diacetyl reaction is carried out on it. A solution of Witte's peptone which gave a well-marked red coloration and green fluorescence with diacetyl and alkali was submitted to prolonged digestion with trypsin. On testing the mixed products of hydrolysis by the diacetyl reaction, it was found that while a red colour was given as before, there was no fluorescence. That the constituent of the protein responsible for the fluorescence had not been broken down or removed by the digestion was readily shown. To the tryptic digest a few drops of the alkaline solution of the colouring matter derived from dicyandiamide were added, and the fluorescence was immediately obtained. It follows, therefore, that hydrolysis of the protein destroys its fluorescent power, not by removing the constituent responsible for the fluorescence, but by altering the form in which the guanidine nucleus is present, the result being that with unhydrolysed protein a colouring matter is produced with diacetyl which is capable of causing fluorescence, while with hydrolysed protein, the arginine which is split off from the protein molecule yields, as we have already seen, a colouring matter which is incapable of producing fluorescence with the active constituent of the protein molecule. It would also seem that arginine, as combined in the protein molecule, has a different constitution from arginine as split off by hydrolysis, since in the first case it gives a fluorescence producing colouring matter with diacetyl, and in the second case a colouring matter which is not fluorescence-producing. An alternative, though less probable possibility is that protein may contain in addition to arginine another guanidine derivative, which gives with diacetyl a colouring matter capable of fluorescing with the active constituent of the protein, but that on hydrolysis this derivative is destroyed or modified.

The fraction of the protein responsible for the fluorescence has not been determined, but it may be pointed out that different proteins differ considerably in their fluorescing power. The power of a protein to fluoresce with these colouring matters may be determined by adding a few drops of the alkaline solution of the colouring matter to a solution of the protein to be tested in the manner previously described. When tested in this way it was found that of four well-known brands of peptone Witte's showed the most marked fluorescence, while gelatine and blood-serum were also very active. On the other hand, a solution of ox heart protein was almost completely inactive. It seems probable that the chemical group in the protein responsible for the fluorescence is contained either in an amino-acid or else in some relatively simple polypeptide, in view of the fact that prolonged tryptic digestion did not destroy it. A number of the more accessible amino-acids such as alanine, arginine, aspartic acid, cystine, glycine, leucine, tryptophane and tyrosine may be definitely excluded.

THE REACTION WITH ACETOIN.

We may now pass from the reaction with diacetyl to the Voges-Proskauer reaction or the reaction with acetoin. Harden (1906) believed that acetoin

was readily converted into diacetyl in alkaline solution by atmospheric oxygen, and that this conversion was the first step in the Voges-Proskauer reaction. His chief reason for this belief was that the colour began to appear at the top of the tube, indicating that oxidation was a factor in the reaction. This reason, however, cannot be accepted as valid, since, as has been shown above, oxygen is just as essential for the reaction with diacetyl as for the reaction with acetoin, and with careful observation the reaction may be seen to commence at the top of the tube in both cases. In consequence, the behaviour of acetoin in alkaline solutions in air was studied to see if any evidence could be found for the conversion of acetoin into diacetyl in these circumstances.

Acetoin was synthesized by the method of Henry (1900) as developed by van Reymenant (1900). During the course of the synthesis it was found that chlor-ethyl methyl ketone ($CH_3.CO.CHCl.CH_3$) reacts with dicyandiamide to give a red-coloured body just as does acetoin. Acetoin was found to be remarkably stable even in strong alkali with free exposure to air. In 20 per cent. caustic soda solution it deepened in colour and ultimately gave a brownish solution, but the change was slow, and acetoin was still readily detectable in the solution after two days. Had a rapid conversion to diacetyl taken place the latter would have been converted to *p*. xyloquinone, which would have turned almost black and acquired the characteristic odour. Instead the brownish residue formed has an odour rather reminiscent of diacetone alcohol. When the change is complete, on warming the tube a camphoraceous odour is obtained. From this simple observation it would appear that in alkaline solutions in air acetoin undergoes a form of condensation with itself and is not converted into diacetyl. The matter was tested further. A strong solution of acetoin in ether was poured on top of one-fifth of its volume of 20 per cent. caustic soda in a small flask with free access to air, and the flask was vigorously shaken from time to time, being allowed to stand between the shakings. The ether was removed and evaporated in a current of air, but no *p*.xyloquinone could be detected in the residue after evaporation either by its odour or by the very delicate test of adding alkali previously mentioned. The time allowed for the experiment varied from three hours to a week, but in no case was any *p*.xyloquinone formed. That oxygenation was sufficient in the circumstances of the experiment was shown by the fact that dicyandiamide and alkali covered with an ethereal solution of acetoin gave a red colour of great intensity in a very short time on agitation. The above result with a strong ethereal solution of acetoin is in marked contrast to what is obtained with even a very weak ethereal solution of diacetyl in the circumstances of the above experiment. Using a very weak solution of diacetyl *p*.xyloquinone could be detected with the greatest of ease in the residue after evaporating the ether. The conclusion appears justified that acetoin is not converted into diacetyl by atmospheric oxygen in alkaline solutions, and it is therefore probable that oxidation of acetoin to diacetyl is not the first step in the Voges-Proskauer reaction.

This conclusion is verified by the following observation: 0·1 gm. of dicyandiamide was dissolved in 2 c.c. of water in each of two similar tubes. Five drops of 1 per cent. diacetyl solution were added to one of the tubes and 5 equal drops of 1 per cent. acetoin to the other. The solutions of diacetyl

and acetoin were, for all practical purposes, equimolecular. After mixing the reagents, 2 c.c. of 40 per cent. caustic soda were added to both tubes, which were well shaken, and placed in a beaker of water at room temperature. They were allowed to remain at constant temperature in this way for four hours, being shaken vigorously from time to time. At the end of this period it was found by the colorimeter that the intensity of the colour produced with acetoin was five-sixths of that produced with diacetyl. The dicyandiamide was shown to be in excess in both tubes. Were the acetoin to have been converted into diacetyl as a preliminary to colour production, this result would mean that what is virtually a quantitative conversion had taken place in four hours. In view of the many ways in which loss would occur, as by condensation of acetoin with itself, and in view of the fact that no *p*.xyloquinone, indicating conversion of acetoin into diacetyl, is formed from acetoin in alkaline solution in the presence of air, it seems evident that the reaction cannot proceed by the conversion of acetoin into diacetyl as a preliminary.

The possible alternatives to this course are very numerous, and their investigation presents difficulties even greater than those attending the study of the reaction with diacetyl, owing to the stability of acetoin in alkali. It may be that the acetoin undergoes enolization as a preliminary to condensation with the guanidine derivatives, or that it undergoes polymerization. The exact stage at which oxidation enters the reaction cannot be determined by the simple experiment used for this purpose in studying the behaviour of diacetyl with the guanidine derivatives, because, owing to the manner in which acetoin resists the action of alkali, the possibility of residual acetoin being responsible for colour production on admitting air to the tube cannot be ruled out. In other words, the exact mechanism of the reaction with acetoin will only be determined by isolation and analysis of the intermediate and final products of the reaction.

NATURE OF SUBSTANCES REACTING WITH ACETOIN.

The substances which react with acetoin are the same as those which react with diacetyl, and what has been said about their behaviour with this body is also true of their behaviour with acetoin, both as regards their constitution, the colours produced, the formation of magnesium lakes by these colours and the formation or absence of fluorescence. It would therefore seem that the final products of the reactions are the same whether diacetyl or acetoin is employed. The sole difference in behaviour is the rate at which the colour develops. With diacetyl the formation of colour is always more rapid than with acetoin, although the intensity is little greater with diacetyl than with acetoin, if sufficient time be permitted to elapse for the reaction with the latter to reach completion. The velocity of the reaction, however, with different guanidine derivatives varies according to the guanidine derivative used, just as in the case of diacetyl. With creatine, for example, the colour production is very much more rapid than with dicyandiamide, and this fact has been utilized by me (1931) as a rapid means of testing for the formation of acetoin by bacteria.

DISCUSSION.

Certain facts emerge from consideration of the results recorded above which are at variance with the usual views held with regard to the nature of the Voges Proskauer reaction and the diacetyl reaction with protein. In the first place, evidence is lacking that the first step in the Voges-Proskauer reaction is an oxidation of acetoin to diacetyl. Such evidence as has been obtained on this point suggests strongly that oxidation of acetoin to diacetyl does not occur as part of the reaction. The exact stage at which oxidation comes into the Voges-Proskauer reaction is not easily determined, but in dealing with the diacetyl reaction matters are much clearer. The first step in this reaction is a condensation between diacetyl and certain guanidine derivatives with the formation of a colourless body, which is subsequently oxidized rapidly by the oxygen of the air to form a red colouring matter. The guanidine derivatives which are known to give a red colour with acetoin and diacetyl may be expressed by the general formulæ HN : C. NH_2NHR and HN : C. NH_2NRR_1, in accordance with current views as to the constitution of such bodies. The end-products of the reactions which take place between substances of this type and acetoin are the same as those obtained with diacetyl. Not all the colouring matters are of the same nature, however, since their chemical reactions are different, one of the most striking differences being the power of certain of them to form well-defined magnesium lakes—a power which others do not possess. One substance, melamine, which does not belong to the guanidine group, gives a reaction to all intents identical with that given by dicyandiamide.

The fluorescence observed in the Voges-Proskauer reaction and in the diacetyl reaction, when proteins are used as the test substances, is not attributable to union of diacetyl with a constituent of unhydrolysed protein, as has been held up to the present. It is, in reality, produced by an interaction which takes place between the colouring matter produced from the guanidine fraction of the protein and some constituent of the protein molecule which has not been identified. The power of the coloured bodies produced from guanidine derivatives to give the fluorescence with protein runs parallel to their power of forming magnesium lakes. All proteins are not equally rich in the group responsible for fluorescence with the active colouring matters, and the group appears to be contained in some relatively simple end-product of tryptic digestion of protein.

From the results described above the reason is now apparent why attempts to hasten the Voges-Proskauer reaction by the use of oxidizing agents have not proved successful in practice. The use of oxidizing agents has been suggested on the assumption that acetoin is first oxidized to diacetyl as part of the reaction. This assumption has been shown to be unjustified, and it is probable that oxidation enters into the reaction at a later stage when the colour is being formed. The colour is, however, readily bleached by oxidizing agents, and in consequence an oxidizing agent added for the purpose of hastening the reaction would only be successful if it were selective in that it hastened the colour formation more than the colour destruction. The only selective oxidizing agent found for this purpose up to the present is molecular oxygen.

SUMMARY.

1. Oxygen is just as essential for colour production in the diacetyl reaction as in the Voges-Proskauer reaction, and the weight of evidence is against the view that acetoin is oxidized to diacetyl as the first step in the latter reaction.

2. The substances which give a red colour with acetoin or diacetyl may, with the exception of melamine, be represented by the general formulæ $HN : C.\ NH_2NHR$ and $HN : C.\ NH_2NRR_1$.

3. The red colouring matters produced do not all exhibit the same chemical properties but fall into well-defined groups.

4. The fluorescence observed in the Voges-Proskauer reaction and in the diacetyl reaction when protein is used is due to an interaction between the colouring matter and some undetermined constituent of the protein molecule.

5. The reason why hydrolysed protein does not give the fluorescence is explained.

6. Arginine shows certain anomalies in its behaviour which suggest that it is not correctly represented by its present formula, and that it is linked in protein in a different form to that obtained on hydrolysis of the protein.

7. The failure of oxidizing agents to hasten the Voges-Proskauer reaction is explained.

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